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THE INFLUENCE OF GLYCEROL ON THE SKIN NECROSIS PRODUCED BY STAPHYLOCOCCUS TOXIN.

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STAPHYLOCOCCUS *a* toxin, when injected into the epilated skin of rabbits or guinea-pigs, produces a characteristic necrotic lesion. The necrotic activity of a toxic filtrate can be approximately determined by estimating the highest dilution that will produce necrosis in the skin. Less toxin than the minimal necrotic dose, so determined, will produce no necrosis; larger amounts will cause necrotic lesions of increased size.

If the toxin is made up in a diluent containing glycerol, the area of the necrosis produced is diminished.

Example.—Into the epilated skin of the left flank of a guinea-pig was injected a series of mixtures containing a constant amount of staphylococcus toxin (3 minimal necrotic doses) and graded amounts of glycerol. Injections of the same amount of toxin without glycerol were made into corresponding sites on the right flank, to control variation in sensitivity over different parts of the skin. The glycerol was measured in undiluted form by means of a micrometer syringe (Trevan, 1925), toxin was then added and the mixtures were brought to constant volume by the addition of saline. The result is shown in Table I.

TABLE I.—*Showing the Inhibition Caused by Glycerol of the Necrotic Action of Staphylococcus Toxin.*

Volume glycerol injected (c.c.).	Percentage glycerol in mixture.	Dose toxin injected (c.c.).	Total volume injected (c.c.).	Skin site.	Diameters of necrosis (mm.).
0·033	16·5	0·01	0·2	1	2 × 2
0·016	8·0	,,	,,	2	2 × 12
0·008	4·0	,,	,,	3	8 × 18
0·0033	1·65	,,	,,	4	10 × 20
0·0016	0·8	,,	,,	5	14 × 22
0	0	,,	,,	6	22 × 25
,,	,,	,,	,,	7	22 × 22
,,	,,	,,	,,	8	22 × 25
,,	,,	,,	,,	9	25 × 25
,,	,,	,,	,,	10	22 × 22

Skin site No. 1 was comparable in position to No. 6, No. 2 to No. 7 and so on. It is clear that inhibition occurs to some extent even when the concentration of glycerol is as low as 0·8 p.c.

The Influence of Glycerol in Antitoxin Assays.

The necrotic action of staphylococcus toxin is commonly utilized in the assay of staphyloccoccus antitoxin. The larger the number of reacting doses of toxin present in the mixtures injected, the more precise the assay; that is to say, concentrated mixtures permit of a greater precision of testing than dilute ones. For a high degree of precision, the mixtures injected should contain at least 25 necrotic doses of toxin. The International unit of staphylococcus antitoxin will neutralize approximately 50 skin-necrotic doses of a typical toxin, so that it is desirable to use at least 0·5 unit in each mixture to be injected. The International and British standard antitoxins are issued periodically in the form of a 66 p.c. glycerol-saline solution, containing 20 units per c.c. The amount of standard antitoxin solution that contains 1 unit will thus contain 0·033 c.c. glycerol. If this amount is injected in a total volume of 0·2 c.c., the glycerol concentration of the injected mixture is 16·5 p.c.—a concentration sufficient to inhibit strongly the necrotic action of the toxin. Even with mixtures 10 times more dilute, inhibition will be noticeable.

It will thus happen that when an antitoxin without glycerol is assayed in comparison with the glycerinated standard by the intracutaneous method, its potency will be assessed at too low a figure. This is illustrated in Table II, in which are compared the anti-necrotic activities of two samples of the British standard antitoxin, one made up in 66 p.c. glycerol and the other in saline. Corresponding mixtures were injected into corresponding sites on opposite flanks of the same guinea-pig.

TABLE II.—*Showing a Comparative Titration of the British Standard Antitoxin With and Without Glycerol.*

Units AT injected.	Dose glycerol injected (c.c.).	Dose toxin injected (c.c.).	Total volume injected (c.c.).	Diameters of necrosis (mm.).
0·83	0·027	0·1	0·2	2 × 2
0·91	0·030	,,	,,	1 × 2
1·00	0·033	,,	,,	No necrosis
1·10	0·036	,,	,,	,,
1·21	0·040	,,	,,	,,
0·83	0·000	,,	,,	18 × 30
0·91	,,	,,	,,	18 × 22
1·00	,,	,,	,,	13 × 20
1·10	,,	,,	,,	8 × 16
1·21	,,	,,	,,	No necrosis

For some time it had been observed that assays of staphylococcus antitoxin by the intracutaneous method frequently resulted in lower values than were obtained by the hæmolytic method. In these comparisons the standard antitoxin was glycerinated, but the sera under test were diluted in saline. The discrepancies can be largely eliminated by using a solution of standard antitoxin made up without glycerol, as is shown in Table III.

TABLE III.—*Titration of Staphylococcus Antitoxic Sera in Comparison with the International Standard Antitoxin.*

Serum.	Hæmolytic method (Lh).	Intracutaneous method ($Lr/_2$).	
		Standard without glycerol.	Standard glycerinated.
"Abyss"	400	400	280
18	290	280	210
6–1	330	315	230
B	87	90	70

(Figures denote International units per c.c. serum; the same toxin was used for all determinations.)

The present method of issue of standard serum is thus unsuitable if the standard is to be used for direct comparison in intracutaneous titrations in the guinea-pig. For such titrations, the standard for comparison should be used in a diluent which contains no glycerol.

Specificity of the Reaction.

The inhibiting influence of glycerol appears to be specific for staphylococcus toxin. No such inhibition could be shown when toxins of *C. diphtheriæ*, *Cl. histolyticum* or of *Vibrion septique* were similarly investigated. It is possible that glycerol causes a slight inhibition of the necrosis produced by the toxin of *Cl. welchii*, but the reactions produced by this toxin in the guinea-pig's skin are very variable in size, and the effect, if any, is difficult to ascertain with accuracy. It is, in any case, much less than with staphylococcus toxin.

Compounds with a chemical constitution similar to that of glycerol exert a similar influence on staphylococcus toxin. Ethylene glycol, sucrose and glucose, all of which contain a high proportion of hydroxyl groups in their molecules, inhibit the necrotic action of the toxin. The inhibition appears to be related to the hydroxyl content rather than to the molecular weight. It is thus not an osmotic effect. Neither agar nor gelatin exert any inhibition.

The Mechanism of the Inhibition.

For this investigation ethylene glycol was sometimes used instead of glycerol. Its inhibiting influence on the necrosis is equal to that of glycerol, it is less toxic and less difficult to work with. The question as to whether the toxin is actually destroyed by the hydroxyl compound was investigated. The following results of experiments bear on this point:

(i) The reaction is apparently instantaneous; it does not progress with a longer time of contact between the hydroxyl compound and the toxin.

(ii) Titrations of staphylococcus antitoxin by the hæmolytic method, as ordinarily carried out, are unaffected by the presence of glycerol. It should be pointed out, however, that in hæmolytic titrations the total volume is 2·5 c.c., whereas in intracutaneous titrations the total volume is only 0·2 c.c. Thus, the glycerol *concentration* is twelve times greater in the latter method. Equal concentrations of glycerol inhibit the hæmolytic and necrotic actions of the toxin to approximately equal degrees.

(iii) Ethylene glycol diminishes the toxicity of staphylococcus toxin for the rabbit. Two mixtures were made up, the first containing 3·3 c.c. ethylene glycol and 0·8 c.c. toxin, and the second 3·3 c.c. borate buffer solution and 0·8 c.c. toxin. The mixtures were stored overnight, then diluted in saline and injected intravenously into groups of rabbits, each rabbit receiving 0·12 c.c. toxin (2 lethal doses) in a total volume of 5 c.c. All the 5 rabbits which received the toxin-glycol mixture survived 5 days. Of the 5 rabbits receiving toxin in borate buffer 1 survived, but 4 died within 4 hours of injection. The difference in survival rates between the two groups is statistically significant.

(iv) The reduction in toxicity persists after the bulk of the glycol has been removed by dialysis. Toxin-glycol and toxin-saline solutions were tested, both intracutaneously in guinea-pigs and intravenously in rabbits, before and after pressure-dialysis for 24 hours. The toxin-glycol mixtures proved significantly less toxic than the controls without glycol by both methods, even after dialysis.

(v) Glycol does not protect rabbits against the lethal action of staphylococcus toxin if it is injected 5 minutes before the toxin.

It thus appears that the action of the glycol is one of partial destruction of the toxin.

An attempt to determine whether the antigen is destroyed or whether the toxin is merely converted to toxoid gave inconclusive results. Groups of guinea-pigs were immunized with toxin-glycol and toxin-saline mixtures. While the mean antitoxin production was slightly lower in the group receiving the toxin-glycol mixture, it was not significantly so.

SUMMARY.

1. Glycerol inhibits the necrosis produced by staphylococcus toxin in the skin of guinea-pigs.

2. The low values sometimes obtained when staphylococcus antitoxin is titrated by the intracutaneous method are attributable to the use of a standard serum diluted in glycerol.

3. The inhibiting action of glycerol is specific for the toxin of the staphylococcus.

4. Inhibition is also produced by ethylene-glycol, sucrose and glucose.

5. The action is one of partial destruction of the toxin.

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CENTRIFUGATION STUDIES: IV. THE MEGATHERIUM BACTERIOPHAGE AND THE VIRUSES OF EQUINE ENCEPHALO-MYELITIS AND LOUPING ILL.

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THE series of centrifugation studies with phages and viruses (I. Elford, 1936; II. Elford and Andrewes, 1936; III. Elford and Galloway, 1937) has been extended to include the megatherium bacteriophage (de Jong) and the viruses of equine encephalo-myelitis and louping ill, thus permitting further comparison of the probable particle sizes of the respective active agents indicated by centrifugation with their values already obtained by ultra-filtration.

The "Ecco" high-speed centrifuge and the inverted capillary tube technique described in the first paper of the series (Elford, 1936) have been used throughout.

MEGATHERIUM BACTERIOPHAGE.

This bacteriophage was very kindly given to us by Dr. L. E. den Dooren de Jong, to whom we express grateful acknowledgment. The phage is produced in broth cultures of the spore-bearing lysogenic strain *B. megatherium* 899. Having obtained the phage from such a culture, we subsequently prepared our stocks of phage in cultures of the mutilate strain, *B. megatherium* 338 C, which was also used in the agar plate tests. In all cases the lysed cultures were filtered through membranes of average pore diameter (A.P.D.) 0·8μ to remove completely any resistant organisms.

Comparison of the merits of broth + Ringer, broth + saline, and broth + $M/45$ phosphate buffer (each a 50 : 50 mixture at pH 7·6) as media for the production of the phage in the presence of the susceptible organism showed that the broth + Ringer system was the best. This accords with the findings of Wollman and Wollman (1936), who found the presence of Ca in the medium to be definitely beneficial for the production of this phage. Unless otherwise stated, the medium for all our phage preparations has been a mixture of equal parts of Hartley's broth and Ringer solution at pH 7·4–7·6. The stock membrane filtrates have possessed phage contents ranging from 10^7 to 10^8 particles per c.c. indicated by the plaque count method. Filtrates through Seitz filters were much less active, due presumably to loss of phage by adsorption.

In making the plaque count estimation, a 6-hours-old broth culture of *B. megatherium* 338 C was always used, 1 drop of the turbid culture being deposited on a 2 p.c. agar plate together with 0·1 c.c. of the phage dilution. After spreading and drying, the plate was incubated at 37° C. overnight. Such a uniform procedure is essential to ensure consistent results. The mean of counts made on at least 3 and sometimes 6 plates was taken.

Ultra-filtration Experiments.

Stock membrane filtrates of the phage in broth + Ringer medium were filtered through collodion membranes, of graded porosities, made according to the method of Elford (1931). The phage was completely retained by membranes of A.P.D. less than 90 mμ. This is interpreted (see Elford, 1933) as indicating that the particle diameter of this megatherium phage is 30–45 mμ. Levaditi, Païc, Voet and Krassnoff (1936), using a series of membranes similar to ours, have also found the size of this same strain of megatherium phage to be 30–40 mμ.

Purified phage.—Phage contained in a stock membrane filtrate having a titre $1{\cdot}7 \times 10^8$ particles per c.c. was washed on a 66 mμ. membrane (*a*) with Ringer solution as medium and (*b*) with physiological saline, until free of demonstrable protein (sulphosalicylic acid test). The titres of the washed preparations of phage were (*a*) $4{\cdot}4 \times 10^7$/c.c. and (*b*) $4{\cdot}9 \times 10^7$/c.c. Subsequent tests on the purified phage indicated that while it kept better in Ringer solution than in saline, its stability was definitely less than that shown by the control unwashed phage in the broth + Ringer medium.

Centrifugation Experiments.

Purified and unpurified preparations of the phage were studied, in each case a hundredfold dilution being made in broth of known density and viscosity. The results of the centrifugation experiments are summarized in Table I. The particle diameter d in mμ. is calculated from the relationship—

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta \log. \dfrac{x_1 + l}{x_1 + l\, C_t/C_o}}{(\sigma_p - \sigma_m)\, N^2\, t}} \quad . \quad . \quad . \quad (1),$$

where σ_p and σ_m are the densities of the particle and medium respectively, η = the viscosity of the medium, x_1 = the distance of the top of the capillary from the centre of rotation, l = length of the capillary, N = speed of centrifuge in r.p.m., t = time of centrifugation in minutes, and C_t/C_o is the ratio of the average concentration C_t within the capillary after spinning for time t to the concentration C_o in the unspun control. No direct measurements have been made of the specific gravity of this megatherium phage, but in calculating the values of d in Table I it has been assumed that $\sigma_p = 1{\cdot}25$. This is the value found in earlier studies for the density of a staphylococcus bacteriophage (Elford, 1936).

The results in Table I indicate the particle size of this megatherium phage to be 30–37 mμ., in good agreement with the value obtained by ultra-filtration.

TABLE I.—*Centrifugation Data for Megatherium Bacteriophage.*

Experiment.	Cell.	x_1 (cm.)	Medium.	σ_m.	η.	Speed (r.p.m.).	Time (min.).	C_o (particles per c.c.).	C_t (particles per c.c.).	C_t/C_o.	Particle diameter d in mμ.	Remarks.
1	M. 0·2	7·25	Broth	1·012	0·0104	10,000	90	$1·8 \times 10^6$	$3·4 \times 10^5$	0·19	37	Experiments 1 and 2 with unwashed phage and metal cells with 0·2 cm. capillaries (M. 0·2).
	,,	,,	,,	,,	,,	,,	,,	,,	$3·6 \times 10^5$	0·20	37	
2	,,	7·25	Broth	1·014	0·0107	10,000	90	$5·8 \times 10^5$	$2·9 \times 10^5$	0·49	30	
	,,	,,	,,	,,	,,	,,	,,	,,	$2·3 \times 10^5$	0·40	32	Experiment 3 with washed phage and glass capillaries 0·2 cm. and 0·3 cm. diameter (G. 0·2 and G. 0·3). In all cases the length of the capillary was 1 cm.
3	G. 0·2	6·75	Broth	1·014	0·0107	10,000	105	$2·5 \times 10^5$	9×10^4	0·36	32	
	G. 0·3	,,	,,	,,	,,	,,	,,	,,	$9·5 \times 10^4$	0·38	31	

TABLE II.—*Centrifugation Data for the Virus of Equine Encephalo-myelitis (N.J. strain).*

Source of virus.	Speed (r.p.m.).	Period (min.).	Titre of unspun control. Dilution.	Titre of unspun control. Test.	Titre after spinning. Dilution.	Titre after spinning. Test.	C_t/C_o.	Remarks.
Guinea-pig brain	11,000	180	..	..	10^{-2}	+−	Nearly 0·01	1. In each experiment a stock membrane (0·7μ) filtrate was diluted 1 : 9 in a broth of density 1·012 and viscosity 0·0104.
			10^{-3}	++	10^{-3}	−−		
			10^{-4}	−−	10^{-4}	−−		
Ditto	10,800	182	10^{-3}	++	10^{-2}	++	0·01	2. Metal cells each with seven capillaries 1·5 mm. diameter and 1 cm. in length.
			10^{-4}	++	10^{-3}	−+		
			10^{-5}	−+	10^{-4}	−−		
			10^{-6}	−−	10^{-5}	−−		
Egg membrane	11,000	180	10^{-5}	++	10^{-3}	++	0·01	3. All tests were by intracerebral inoculation into guinea-pigs.
			10^{-6}	−−	10^{-4}	−−		
					10^{-5}	−−		
Guinea-pig brain	10,000	135	10^{-5}	++	10^{-4}	++	0·1 to 0·01	4. + means animal died of the disease. − means animal remained normal.
			10^{-6}	+−	10^{-5}	−−		
			10^{-7}	−−	10^{-6}	−−		
Egg membrane	10,000	135	10^{-6}	++	10^{-6}	++	1 to 0·1	5. All animals remaining normal were tested for immunity but none was found to be immune.
			10^{-7}	+−	10^{-7}	−−		
Guinea-pig brain	10,800	120	10^{-5}	++	10^{-3}	++	0·1 to 0·01	
			10^{-6}	−−	10^{-4}	+−		
					10^{-5}	−−		
Ditto	11,500	95	10^{-4}	++	10^{-4}	+−	0·1	
			10^{-5}	+−	10^{-5}	−−		
			10^{-6}	−−	10^{-6}	−−		

It is interesting to note that both the purified and unpurified preparations of the phage were found to have the same particle size.

EQUINE ENCEPHALO-MYELITIS VIRUS.

Two strains of the equine encephalo-myelitis virus have been studied during this work, (1) the western strain S.D. (South Dakota, 1932) as used by Kelser (1933) and Ten Broeck and Merrill (1933–4), (2) the eastern strain N.J. (New Jersey and Virginia, 1933). Both strains, which have been maintained by cerebral passage in guinea-pigs, were kindly sent to us from America by Dr. E. W. Hurst, who has since employed them in experiments with monkeys and guinea-pigs at the Lister Institute (Hurst, 1936).

Source of Virus and Method of Testing.

The brain tissue of infected mice and guinea-pigs, and also the chorio-allantoic membranes of the 25th to 30th culture passages in hen's eggs, have served in turn as the source of virus. 2·5 p.c. suspensions of the ground-up tissue in broth, after preliminary clarification by centrifugation and filtration through asbestos or sand and pulp, were filtered through suitable graded membranes to provide bacteria-free stock filtrates. In testing for the presence of the virus, serial tenfold dilutions of the liquids were made and inoculated intracerebrally into 2 guinea-pigs (0·25 c.c. dose) or into 2 to 6 mice (0·01 c.c. dose), while in some experiments 0·05 c.c. amounts were placed on the chorio-allantoic membrane of the developing hen's egg by the technique of Burnet and Galloway (1934). Comparison of the sensitivities of these three methods in testing for virus potency has shown no one of them to be appreciably more sensitive than any other. Certainly no differences have been found as great as those observed with vesicular stomatitis virus, which, when titrated on the egg membrane, exhibits a titre 100 to 1000 times that indicated by the inoculation of the pad of the guinea-pig.

Ultra-filtration Experiments.

Bauer, Cox and Olitsky (1935) found that the virus from infected mouse brain and also from infected tissue cultures of chick embryo suspended in broth was able to pass gradocol membranes of A.P.D. 66 mμ. but was completely retained by 60 mμ. membranes. This indicated that the particle size of the virus is 20–30 mμ. Similar ultra-filtration experiments by one of us (F. F. T.), using virus from the three sources mentioned above, have shown the filtration end-point to be rather nearer 70 mμ. than 60 mμ., with the corresponding particle size 25–35 mμ. This is in good agreement with the findings of the American workers, and the evidence of ultrafiltration may be summarized as indicating a probable particle size of 20–35 mμ.

Centrifugation Experiments.

Centrifugation studies have been made upon stock membrane filtrates of the eastern N.J. strain of equine encephalo-myelitis virus from infected guinea-pig brain and also egg membrane cultures. The results are given in Table II.

The virus from both sources behaved similarly. In a broth medium of density 1·012 and viscosity 0·0104, the average concentration within capillary cells 1 cm. in length and 0·15 cm. diameter was reduced to one-tenth the original by spinning for 120 minutes at 11,000 r.p.m. As no measured value is available for the density of the virus, the particle diameter has been calculated for the two probable extreme values of σ_p. Thus by relationship (1)—

if $\sigma_p = 1{\cdot}20$ then the particle diameter $= 39$ mμ.
$\sigma_p = 1{\cdot}30$,, ,, ,, $= 32$ mμ.

These figures support the ultrafiltration results, and it is interesting to note that the agreement between the findings of the two methods becomes the more complete as the density of the virus is assumed to approach that of protein substance, viz. 1·30–1·35.

LOUPING-ILL VIRUS.

The virus of louping ill (sheep) has previously been shown by ultrafiltration to have a probable particle size of 15–20 mμ. (Elford and Galloway, 1933). In view of the successful centrifugation experiments with the still somewhat smaller virus of foot-and-mouth disease, we decided to acquire additional evidence on the centrifugation behaviour of yet another of the smallest viruses. Louping-ill virus seemed the obvious choice.

The strain of virus used, the preparation of the stock bacteria-free filtrates and the method of testing for the virus were exactly as described in the earlier paper on ultrafiltration studies, to which reference has already been made.

In the centrifugation experiments metal cells were used each containing 19 capillaries 1 mm. in diameter and 0·5 cm. in length, as employed in the studies with the virus of foot-and-mouth disease (Elford and Galloway, 1937). Careful control tests were made to determine whether or not the metal exerted any deleterious action on the virus during such periods of contact as occurred in these studies. No harmful effect could be detected. In all experiments the virus was contained in a broth medium. The stock membrane filtrate was diluted 1 : 9 in broth of density 1·014 and viscosity 0·011. The results of our centrifugation experiments are given in Table III. A tenfold diminution

TABLE III.—*Centrifugation Data for the Virus of Louping Ill.*

Experiment.	Speed (r.p.m.).	Period (min.).	Unspun control. Dilution.	Unspun control. Test.	After spinning. Dilution.	After spinning. Test.	c_t/c_o	Remarks.
1	10,000	120	10^{-2}	++	..	..	0·1	1. The medium was in each case broth at pH 7·4, having density 1·014 and viscosity 0·011.
			10^{-3}	++	10^{-2}	++		
			10^{-4}	−−	10^{-3}	−−		
			10^{-5}	−−	10^{-4}	−−		
2	10,000	120	10^{-2}	++	10^{-2}	+−	0·1	2. A stock membrane filtrate (0·38μ) was used in each experiment.
			10^{-3}	+−	10^{-3}	−−		
			10^{-4}	−−	10^{-4}	−−		
3	10,000	180	10^{-2}	++	10^{-1}	+−	0·01	
			10^{-3}	+−	10^{-2}	−−		
			10^{-4}	−−	10^{-3}	−−		
4	10,000	90	10^{-2}	++	10^{-2}	++	1	
			10^{-3}	++	10^{-3}	++		
			10^{-4}	−−	10^{-4}	−−		

in the average concentration of virus in the cell was effected by spinning for 120 minutes at 10,000 r.p.m.

Now by relationship (1), with values for these experiments: $x_1 = 7{\cdot}75$, $l = 0{\cdot}5$ and σ_m and η are as quoted above, and $C_l/C_o = 0{\cdot}1$.

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{0{\cdot}011 \log. \frac{8{\cdot}25}{7{\cdot}80}}{(\sigma_p - 1{\cdot}014)\,(10{,}000)^2\,120}}$$

If $\sigma_p = 1{\cdot}20$ then $d = 27\ m\mu$.
$\sigma_p = 1{\cdot}30$,, $d = 22\ m\mu$.

DISCUSSION.

The evidence of the foregoing centrifugation studies with the megatherium bacteriophage and the viruses of equine encephalo-myelitis and louping ill lends general support to the conclusions drawn as to the probable particle sizes of the respective active agents concerned from earlier ultrafiltration work. This is clearly seen in the following summarizing table:

System.	Assumed density of particle.	Particle size by centrifugation (mμ.).	Particle size by ultrafiltration (mμ.).
Megatherium phage	1·25	30–37	30–45
Equine encephalo-myelitis virus	1·20	39	20–35
	1·30	32	
Louping-ill virus	1·20	27	15–20
	1·30	22	

The need for more precise knowledge as to the densities of the particles concerned has once again been emphasized. While we have evidence that some of the largest phages have densities 1·20–1·25 and likewise some of the largest viruses 1·15–1·20, the specific gravities of the smaller viruses have to be based largely on inference. Thus one of the smallest of the known animal viruses, that of foot-and-mouth disease, appears to have a density very like that of protein substance, recent experiments having indicated a value at least as high as 1·30 and maybe as high as 1·40 (Schlesinger and Galloway, 1937). These facts have guided us in taking 1·20–1·30 as being the probable limits for the densities of the two viruses studied here. However, the element of uncertainty on this point must be remembered in assessing the merits of the centrifugation results.

SUMMARY.

The rates of sedimentation of a megatherium bacteriophage and the viruses of equine encephalo-myelitis and louping ill, when under the influence of centrifugal forces, have been studied by the inverted capillary-tube technique. The particle size values indicated for the respective active agents concerned are in general agreement with the earlier values obtained by the method of ultrafiltration analysis.

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THE RELATIONSHIPS BETWEEN LIQUID CRYSTALLINE PREPARATIONS OF CUCUMBER VIRUSES 3 AND 4 AND STRAINS OF TOBACCO MOSAIC VIRUS.

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FOUR viruses affecting cucumber plants have been described by Ainsworth (1935) as cucumber viruses 1, 2, 3 and 4. These fall into two pairs, the individuals of each pair possessing similar general properties. Viruses 1 and 2 are transmitted by aphids, are inactivated by 10 minutes' heating at 60° C., or by a few days' ageing in expressed sap, and are readily transmitted to members of the Solanaceæ and other families. Cucumber viruses 3 and 4 are apparently not transmitted by aphids, are not inactivated by 10 minutes' heating at 80° C., or by some months' ageing in expressed sap, and they have not been transmitted to any plants except members of the Cucurbitaceæ. A further fact which relates viruses 3 and 4 and separates them from 1 and 2 is that cucumber plants infected with virus 3 are resistant to further infection with virus 4, but are still quite susceptible to viruses 1 and 2. Viruses 3 and 4 are differentiated merely because they cause different symptoms. Cucumber plants infected with virus 3 show a general dark green mottle and the leaves

become somewhat blistered and deformed, while those infected with virus 4 show a bright yellow blotchy type of mottling and but little blistering or deformation.

Ainsworth's results suggest that the relationship between viruses 3 and 4 might be of the same type as that between the strains of tobacco mosaic virus, and they also show that the stability of the two viruses *in vitro* is of the same order as that of tobacco mosaic virus. As far as is known the recognized strains of tobacco mosaic virus and cucumber viruses 3 and 4 have no common host plants: we have been unable to infect cucumber plants with three different strains of tobacco mosaic virus; other workers have found the members of the Cucurbitaceæ to be immune to tobacco mosaic virus, and, as already stated, Ainsworth found the host range of cucumber viruses 3 and 4 to be restricted to the Cucurbitaceæ. Comparative infection experiments with the different viruses therefore cannot be made, and it is impossible to determine whether they are sufficiently related to immunize plants against one another. The results presented in this paper show that in spite of the wide differences in the host ranges of the two groups of viruses they are fairly closely related. They have antigens in common, and from cucumber plants infected with viruses 3 and 4 we have isolated nucleoproteins infective at high dilutions (Bawden and Pirie, 1937*a*). These nucleoproteins have similar analytical figures and many properties similar to those previously isolated from solanaceous plants infected with strains of tobacco mosaic virus (Bawden and Pirie, 1937*b*). The differences at present noted between cucumber viruses 3 and 4 and strains of tobacco mosaic virus, however, are considerably greater than those noted between the individual strains of tobacco mosaic virus.

PREPARATION.

The general methods described for the purification of the strains of tobacco mosaic virus have been used for the cucumber viruses, but as the latter proved rather more difficult to isolate, certain modifications of the original method have been made. The final yields obtained have also been less, averaging from 0·2 to 0·3 g. per l. of expressed cucumber sap as compared with about 2 g. with tobacco mosaic virus. Cucumber viruses 3 and 4 precipitate from plant sap at around pH 4·8, whereas the strains of tobacco mosaic virus precipitate at around pH 3·4. This fact possibly accounts for some of the difficulty experienced in freeing the cucumber viruses from plant proteins by precipitation methods. It is also of interest in view of the different host ranges of the two groups of viruses; for the pH value of expressed cucumber sap is between 7 and 8, whereas that of tobacco sap is between 5 and 6. Of the methods of preparation yet tried the following has proved most effective.

Cucumber plants are picked about a month after infection when they are showing most definite symptoms, minced in a meat mincer and the sap expressed through muslin. The mincing is easier and more sap is obtained if the leaves are first sprinkled with a dilute solution of sodium cyanide. This also has two other useful effects: it largely prevents the formation of highly coloured oxidation products which are difficult to remove from the final preparation, and it increases the yield of virus by raising the alkalinity of the extract. 0·5 to 1 g. of cyanide should be used for each kilogram of leaves. The expressed

sap is then heated to 70° C., and rapidly cooled. This treatment produces a flocculent precipitate, which aggregates quickly and is readily thrown down by centrifuging. The precipitate should be thoroughly washed in water and again centrifuged, for a certain amount of virus adheres to it.

The greenish-brown supernatant fluid is then brought to about pH 4·8 by the addition of HCl, and the precipitate produced is centrifuged off. The precipitate is suspended in water and dilute NaOH solution added to bring the pH to about 7·5, when the suspension is centrifuged. Sufficient ammonium sulphate is now added to the clarified supernatant to give a quarter saturated solution, and the precipitate formed is centrifuged off. This precipitate is taken up in water, centrifuged and the precipitate discarded ; the supernatant fluid is then again one quarter saturated with ammonium sulphate. The precipitate obtained at this stage of the preparation is usually greyish in colour, and shows a pronounced satin-like sheen similar to that shown by preparations of tobacco mosaic virus. When examined microscopically (preferably with dark-ground illumination) it is seen to be composed of fairly regularly shaped needles, similar to those which Stanley (1936), working with tobacco mosaic virus, described as crystals. Bernal and Fankuchen (1937), however, have now pointed out that these needles lack the regularity characteristic of true crystals, and that they are ordered in two dimensions only. The precipitate is centrifuged down, dissolved in water and the solution again centrifuged. It easily comes clear by transmitted light, although retaining an intense sheen by reflected light. This fluid is diluted somewhat, and is then brought to about pH 4·8 by the addition of $N/10$ HCl, when a precipitate with the sheen is again produced. This is thrown down and then washed several times by stirring up with water and repeated centrifuging. When the water used for washing is nearly free from sulphate, the precipitate is dissolved by the addition of dilute NaOH solution. At this stage in the preparation of the strains of tobacco mosaic virus it was noticed that the precipitation point with acid showed a definite shift, and the precipitate obtained at pH 3·4 from dilute salt solutions became soluble at this pH when salt-free. This shift is presumably similar to the rather smaller shift in the same direction that has been observed with certain other proteins (Adair and Adair, 1934 ; Smith, 1936). Most preparations of cucumber viruses 3 and 4 have not shown a shift of this type, but a few were found to sediment more easily at about pH 5·5 when salt-free than they did at pH 4·8. In the complete absence of salt the viruses are not readily sedimented by centrifuging at 3500 r.p.m.

At pH 6 preparations of cucumber viruses 3 and 4 are definitely more turbid than are those of tobacco mosaic virus of the same concentration, but the turbidity disappears at above pH 7. Neutral aqueous solutions of the material prepared in this way, especially if young cucumber plants are used as a source of the virus, are sometimes quite colourless, and if more concentrated than about 3 p.c. separate on standing into two liquid layers. More often, however, preparations at this stage are quite definitely coloured and do not layer. Such preparations can only with difficulty be fractionated further by more precipitations with acid and ammonium sulphate. All the material in the solutions is precipitated by these treatments apparently in the paracrystalline form. Such preparations can easily be purified further either by

incubation with trypsin or by high-speed centrifugation. When preparations have been incubated with about 0·2 p.c. trypsin at 38° C. and at pH 8 overnight they have always given colourless neutral solutions which separated into two layers, after two or three further precipitations with acid or ammonium sulphate. Alternatively, the material can be fractionated by centrifuging the neutral solutions at 16,000 r.p.m. This treatment sediments a transparent jelly, which dissolves in water to give a colourless solution that separates into two layers.

The layering phenomenon in purified preparations of cucumber viruses is similar to that previously described with the strains of tobacco mosaic virus (Bawden and Pirie, 1937*b*). It has not been studied in such detail with cucumber viruses 3 and 4, but as yet it has been observed at room temperature only in solutions containing more than 2·5 p.c. of solids. At 0° C. layers can separate from more dilute solutions. The upper layer is the more dilute and by transmitted light is faintly opalescent; the lower layer may be quite clear by transmitted light but generally has an intense sheen by reflected light. When solutions are left undisturbed for some months the lower layer loses the sheen, but it regains it if it is shaken with the upper layer and then again allowed to layer; similarly, if a sample of the lower layer is centrifuged at 16,000 r.p.m. for half an hour a layer of perfectly clear liquid crystalline solution appears in the middle of the tube between the sedimented jelly at the bottom and the very dilute solution remaining at the top. This clear solution shows no sheen. It seems, therefore, that the sheen of the bottom layer is a result of incomplete separation, and is caused by droplets of top-layer fluid suspended in it, and is not a necessary consequence of its liquid crystallinity. The upper layer is not spontaneously birefringent, but readily shows the phenomenon of anisotropy of flow, *i. e.* it becomes birefringent when agitated or when flowing. The jellies which sediment when neutral or acid solutions of cucumber viruses 3 and 4 are centrifuged at high speed are also birefringent.

The sheen and the phenomenon of anisotropy of flow suggest that these particles, like those of tobacco mosaic virus, are rod-shaped, and this has been confirmed by the X-ray measurements of Bernal and Fankuchen (1937).

ANALYSIS.

Dried preparations of cucumber viruses 3 and 4 closely resemble similarly treated preparations of strains of tobacco mosaic virus. When dried in the frozen state the material has a light open texture and is easily handled. After drying the material is still infective, but its ability to show the phenomenon of anisotropy of flow is much reduced and its serological activity is reduced to about one-half. The analytical figures obtained have varied slightly from preparation to preparation, but normally fall in the following ranges:

Carbon	50·00 to 51·0 p.c.
Hydrogen	7·10 „ 7·6 „
Nitrogen	15·30 „ 15·8 „
Sulphur	0·00 „ 0·6 „
Phosphorus	0·55 „ 0·6 „
Carbohydrate	2·20 „ 2·5 „
Ash	1·00 „ 2·0 „

The phosphorus and carbohydrate contents were estimated by the methods previously described (Bawden and Pirie, 1937*b*). The phosphorus and carbohydrate can be isolated as nucleic acid after these viruses have been inactivated by heating, in the same manner as previously described for the strains of tobacco mosaic virus. The intact nucleic acid + protein complex resists attack by trypsin preparations containing nuclease, but when the complex has been disrupted by heat the components are readily attacked by this enzyme mixture. Schmidt (1936) has found other nucleoproteins to behave in this way.

Further evidence that the nucleic acid and protein are combined and not merely mixed in the virus preparations, as Stanley states (1937*a* and *b*), was obtained by analysing the precipitates obtained by centrifuging the virus preparations at a high speed. When solutions of the cucumber viruses are centrifuged at 16,000 r.p.m. at pH 8 and pH 3 birefringent jellies are deposited. After drying, both these have the same nucleic acid content as the material that is obtained by centrifuging at low speeds at the acid precipitation point. Stanley (1937*b*), in an attempt to show that the protein he had isolated was tobacco mosaic virus and not a mixture of protein and virus, centrifuged his products at a number of pH values on either side of the iso-electric point, and showed that the sedimentation rate of the virus (measured by infectivity tests) was the same as that of the protein. From this he argues that the virus is not merely a contaminant adsorbed on to the protein, for if it were it should come off when the pH is sufficiently altered. If this is a valid argument it should also have been possible to separate the nucleic acid from the protein in our experiments by altering the pH if the two components were merely mixed.

The only evidence against our conclusion that the isolated materials are nucleoproteins is in two of Stanley's statements. Firstly (1936), he stated that his preparations of tobacco mosaic virus contained no phosphorus, and secondly (1937*b*), that, as usually prepared, they contain phosphorus and carbohydrate in the form of nucleic acid, but that this is inessential for virus activity and can be removed by prolonged alkaline dialysis. Best (1936) has shown that the infectivity of tobacco mosaic virus preparations is reduced to one half by 12 hours' exposure at room temperature and pH 8·2. Stanley has published no figures to show that the serological activity and infectivity of his virus preparations are unaffected by the treatments used to remove the phosphorus and carbohydrate. In our experience those treatments which lower the phosphorus content to a level at which its detection becomes difficult, *i. e.* 0·05 p.c. or lower, invariably lead to a corresponding decrease in the activity of the virus preparations. The resulting products are still highly infective, *e. g.* infections may be obtained with as little as 10^{-8} or 10^{-9}, but it seems reasonable to assume that these infections are brought about by the remaining, undecomposed nucleoprotein, and not by the dephosphorylated protein which constitutes the bulk of such material.

ACTIVITY AND SEROLOGICAL REACTIONS.

No host plants are known in which cucumber viruses 3 and 4 produce local lesions, and the quantitative methods used for determining the infectivity

of the tobacco mosaic virus preparations at various dilutions could not be employed. The infectivity of the purified cucumber viruses was therefore tested merely by rubbing the leaves of cucumber plants with 1 c.c. of solutions containing various amounts of nucleoprotein, and noting the greatest dilution at which systemic infections were obtained. The results of six tests of this type are shown in Table I, and it will be seen that the smallest amount required to produce infection has varied from 10^{-8} to 10^{-10} g.

TABLE I.—*Infectivity of Purified Preparations of Cucumber Viruses* 3 *and* 4.

Virus.	Dilution.			
	10^{-7}.	10^{-8}.	10^{-9}.	10^{-10}.
Cucumber 3 . . .	++	++	++	−−
,, 3 . . .	++	++	++	+−
,, 3 . . .	++	++	+−	−−
,, 4 . . .	++	++	+−	+−
,, 4 . . .	++	++	++	−−
,, 4 . . .	++	++	−−	−−

The dilution is given in grammes of protein per c.c. of inoculum and two plants were inoculated with 1 c.c. each at every dilution. The + sign indicates systemic infection and the − sign indicates that the plant remained healthy.

Tobacco (var. White Burley), tomato, *Nicotiana glutinosa*, and Golden Cluster beans, which are all susceptible to tobacco mosaic virus at high dilutions, did not become infected when inoculated with solutions containing as much as 1 p.c. of the nucleoproteins isolated from infected cucumber plants.

The purified preparations of cucumber viruses 3 and 4 are antigenic, and antisera precipitating at a dilution of 1 in 1000 were produced by giving rabbits a single intravenous injection of 5 mg. With these antisera the serological titres of the virus preparations were determined by the technique previously described, 1 c.c. of virus solution at various dilutions being mixed with 1 c.c. of antiserum at a constant dilution. The highly purified, liquid crystalline, preparations of cucumber viruses 3 and 4 have given precipitation end points of from $\frac{1}{6 \times 10^6}$ to $\frac{1}{8 \times 10^6}$. The activity is, therefore, very similar to that of purified tobacco mosaic virus, and the serological titres are of the same order as those obtained with other highly purified antigens.

The serological titre of the material as normally prepared gives an accurate index of its infectivity, but certain treatments, *e. g.* with nitrous acid or exposure to X-rays, destroy infectivity without affecting the serological reactions. Preparations of cucumber viruses 3 and 4, rendered non-infective by such methods, still show anisotropy of flow and form liquid crystalline solutions, and when precipitated with acid or ammonium sulphate form paracrystals indistinguishable from those of fully active virus.

The infectivity of our purified virus preparations is not sufficiently great for us to state that they contain only virus particles, and that the nucleoproteins are therefore necessarily the viruses. The high serological titres and data from centrifuging and X-ray experiments suggest that our material is not grossly contaminated, but there remains the possibility that the nucleoproteins

we have isolated are antigens peculiar to the infected plants, and that they are " contaminated " with small amounts of virus responsible for the infections. If this is so, then we must assume that in addition to the viruses themselves multiplying in the infected plants they also produce (or cause to be produced) the nucleoproteins, for these have not been isolated from healthy plants. We have no evidence that infectivity can in any way be dissociated from the nucleoprotein : infectivity is closely correlated with the amount of protein, and loss of infectivity is accompanied by changes in the protein, and denaturation of the protein by heat, acid or alkali is accompanied by loss of infectivity.

At the present stage of the work it seems most reasonable to assume that the nucleoproteins are the viruses, but to remember that the point is not proved, and the possibility still exists that the nucleoproteins are disease products to which the viruses are firmly attached.

Cross-precipitation tests with cucumber virus 3 as antigen and tobacco mosaic virus antiserum, and *vice versa*, have shown that the two viruses are serologically related, for both antigens precipitate with both antisera. The serological titres given by preparations of either virus are practically independent of the antiserum used, but the range of antigen dilution over which precipitation takes place varies greatly with different antisera. When the cucumber viruses are titrated against a constant amount of the antiserum to any of the three strains of tobacco mosaic virus which we have used, there is a central zone of precipitation with large zones of non-precipitation in the antigen excess region (see Table II). When any of the three tobacco mosaic viruses are titrated against a constant amount of antiserum to cucumber virus 3 at the same dilution there are similar zones of non-precipitation in the antigen excess region. When cucumber viruses 3 and 4 are titrated against cucumber virus 3 antiserum, or when any of the three strains of tobacco mosaic virus are titrated against either their own or each other's antiserum, the zones of non-precipitation in the antigen excess region occur only where the antigen is very much more concentrated, and the zones are therefore smaller. The results of an experiment in which tobacco mosaic virus and cucumber virus 3 were titrated against the four antisera are shown in Table II. It will be seen that there are differences in the range of precipitation of the antisera to the individual strains of tobacco mosaic, but these are extremely small in comparison with the differences between the tobacco and the cucumber viruses.

Many virus workers have shown that the individual strains of plant viruses are serologically related, and at the present time it seems that all viruses which have the power of immunizing plants against one another are serologically related. Only Chester (1936) has shown that the strains are not serologically identical. Chester, using clarified infective sap as his antigens, found that he could absorb tobacco mosaic virus antiserum with Aucuba mosaic virus and then get no precipitate with the latter, but still get a precipitate with tobacco mosaic virus. Similarly, if Aucuba mosaic virus antiserum was fully absorbed with tobacco mosaic virus it would still precipitate with Aucuba mosaic virus. Chester also found that some of the strains isolated by Jensen (1933) were serologically identical with tobacco mosaic virus and others with Aucuba

TABLE II.—*Precipitation of Tobacco Mosaic Virus and Cucumber Virus 3 with Different Antisera.*

1. *Tobacco Mosaic Virus as Antigen.*

Antiserum.	Time.	Dilution of antigen (1/1=1 mg. per c.c.).					
		1/1.	1/4.	1/16.	1/64.	1/256.	1/1024.
Tobacco mosaic virus	2 min.	++	++	+	−	−	−
	2 hr.	++++	++++	++++	+++	++	+
	24 ,,	++++	++++	++++	++++	+++	++
Aucuba mosaic virus	2 min.	++	++	+	−	−	−
	2 hr.	++++	++++	++++	+++	++	+
	24 ,,	++++	++++	++++	++++	+++	++
Enation mosaic virus	2 min.	−	++	+	−	−	−
	2 hr.	−	++++	++++	+++	++	+
	24 ,,	−	++++	++++	++++	+++	++
Cucumber virus 3	2 ,,	−	−	−	−	+	+
	24 ,,	−	−	−	+	++	++

2. *Cucumber Virus 3 as Antigen.*

Antiserum.	Time.	Dilution of antigen (1/1=1 mg. per c.c.).					
		1/1.	1/4.	1/16.	1/64.	1/256.	1/1024.
Tobacco mosaic virus	1 hr.	−	−	−	+	++	−
	24 ,,	−	−	−	++	+++	++
Aucuba mosaic virus	2 ,,	−	−	−	−	+	−
	24 ,,	−	−	−	−	++	++
Enation mosaic virus	2 ,,	−	−	−	−	+	−
	24 ,,	−	−	−	−	++	++
Cucumber virus 3	2 min.	+++	++	+	−	−	−
	2 hr.	++++	++++	++++	+++	++	−
	24 ,,	++++	++++	++++	++++	+++	++

In all tests the antiserum was used at a dilution of 1/50. 1 c.c. of antiserum was added to tubes containing 1 c.c. of antigen at given dilutions, and the tubes were immediately placed in a water-bath at 50° C.

+ signs indicate the degree of precipitation.

mosaic virus. Chester does not seem to have considered the possibility of quantitative antigenic differences and his tests seem to have been entirely qualitative ; after the sera had been absorbed, tests for further precipitation were made at only one antigen or antiserum dilution. The tests in which no precipitation was obtained do not therefore necessarily indicate that the serum was completely absorbed, as Chester states, for they may have been made in a region of antigen excess which inhibited precipitation, as shown in Table II. Using purified virus preparations we have made cross-absorption experiments between our virus strains and their antisera, and we have found differences of the same type as those described by Chester. In these tests, as in the straightforward precipitation tests, much greater differences were found between the cucumber and the tobacco viruses than were found between the individual strains of the tobacco viruses. In each test a preliminary experiment was made to determine the optimal combining proportions of the antigen (virus) used for the absorption and the antiserum to be absorbed. The optimal combining proportions were determined by adding 1 c.c. of antigen at different concentrations to each of a series of tubes containing 1 c.c. of serum at a constant dilution. The tubes were immediately placed in a water-bath at 50° C. and the proportions of antigen and antiserum in that tube which first showed a precipitate were taken as optimal (Topley and Wilson, 1936, p. 144). Antigen and antiserum were then mixed with the antigen in slight excess of its optimal value ; the mixture was then incubated for 2 hours at 50° C., placed in the ice-box overnight and then centrifuged. The supernatant fluid was then tested at a constant dilution against the antigen used for absorption at a number of different antigen dilutions. If there was no further precipitation it was then tested against the other virus strains, but if it still precipitated a second absorption was made. The sera, fully absorbed with one virus strain, were then tested for their ability to precipitate with the different virus strains, the tests being made with antiserum at a constant dilution and with antigen varying over a wide range of dilutions.

The amount of virus required to absorb an antiserum varied greatly with the different antigens and antisera : it was greatest when antigens were used to absorb their homologous antisera, somewhat less when the strains of tobacco mosaic virus were used to absorb each other's sera, and very much less when cucumber virus 3 was used to absorb tobacco mosaic virus antiserum or tobacco mosaic virus to absorb cucumber virus 3 antiserum. When tobacco mosaic virus antiserum was absorbed with cucumber virus 3 the precipitation of the serum with tobacco mosaic virus was only slightly affected, and the optimum precipitation point was only slightly shifted. Similarly, when cucumber virus 3 antiserum was completely absorbed with any of the three tobacco mosaic virus strains, its precipitation with cucumber virus 3 was only slightly affected. On the other hand, when antiserum to one strain of tobacco mosaic virus was fully absorbed with another strain its precipitation with the strain used for immunization was greatly affected, and the optimum precipitation point showed a large shift.

In Table III are shown the summarized results of several experiments in which the various antisera were absorbed with different virus strains and then

TABLE III.—*Summarized Results of Cross-absorption Experiments.*

Antiserum absorbed.	Antigen used for absorption.	Precipitation tests with absorbed sera and antigens. Antigen. Tobacco mosaic virus.	Aucuba mosaic virus.	Enation mosaic virus.	Cucumber virus 3.	Cucumber virus 4.
Tobacco mosaic virus	T.M.V.	−	−	−	−	−
	A.M.V.	−	−	−	−	−
	E.M.V.	++	++	−	−	−
	C.V. 3	++++	++++	++++	−	−
	C.V. 4	++++	++++	++++	−	−
Aucuba mosaic virus	T.M.V.	−	++	++	−	−
	A.M.V.	−	−	−	−	−
	E.M.V.	++	++	−	−	−
	C.V. 3	++++	++++	++++	−	−
	C.V. 4	++++	++++	++++	−	−
Enation mosaic virus	T.M.V.	−	+	++	−	−
	A.M.V.	−	−	+	−	−
	E.M.V.	−	−	−	−	−
	C.V. 3	++++	++++	++++	−	−
	C.V. 4	++++	++++	++++	−	−
Cucumber virus 3	T.M.V.	−	−	−	++++	++++
	A.M.V.	−	−	−	++++	++++
	E.M.V.	−	−	−	++++	++++
	C.V. 3	..	..	..	..	..

− indicates that there is no precipitation.
+ signs indicate the degree of precipitation at the optimum. For description of method see text.

tested for their precipitability with other virus strains. From these it is apparent that tobacco mosaic antiserum absorbed with Aucuba mosaic virus contains no residual precipitating antibodies, while when absorbed with Enation mosaic virus it still precipitates with Aucuba mosaic and tobacco mosaic viruses; Aucuba mosaic virus antiserum after absorption with Enation mosaic virus still precipitates with both tobacco mosaic and Aucuba mosaic viruses, and after absorption with tobacco mosaic virus it still precipitates with Enation mosaic and Aucuba mosaic viruses; Enation mosaic virus antiserum after absorption with Aucuba mosaic virus will still give a slight precipitate with Enation mosaic virus but none with tobacco mosaic virus, and after absorption with tobacco mosaic virus it precipitates with both Aucuba mosaic and Enation mosaic viruses.

The results show that in addition to an antigenic fraction common to the three strains of tobacco mosaic virus examined, tobacco mosaic virus and Aucuba mosaic virus contain a fraction not present in Enation mosaic virus, Aucuba mosaic virus and Enation mosaic virus contain a fraction not present in tobacco mosaic virus, and Enation mosaic virus contains a fraction not present in Aucuba mosaic virus. If each fraction distinguished be represented by a letter, then the simplest formulæ for the three strains which can adequately explain the results are:

Tobacco mosaic virus A B.
Aucuba mosaic virus A B C.
Enation mosaic virus A — C D.

It is to be expected that the use of a larger number of strains in the cross-absorption experiments would have shown further differences between the three strains, and it is possible that each of the symbols in the formulæ represent groups of antigens rather than single antigens. If we assume that each of the components has an antigenicity of the same order as the others it would appear that the common fraction A is predominant; for removal of the antibodies to A greatly reduces the power of an antiserum to precipitate. There is, however, no definite evidence on the relative quantities of the different antigens present in these strains.

When tobacco mosaic virus antiserum is absorbed with either Enation mosaic or Aucuba mosaic virus it loses its power of precipitating with cucumber virus 3. The antigens which tobacco mosaic virus shares with cucumber virus 3 therefore must be contained in the common fraction A. The serological relationship between tobacco mosaic virus and cucumber virus 3 is best explained by postulating that, in addition to the antigens specific to each, the two viruses have two common antigens, and that while the total of the two antigens in each is of the same order, tobacco mosaic virus contains a preponderance of one and cucumber virus 3 a preponderance of the other. If we call the common antigens X and Y, then tobacco mosaic virus might be indicated as (NX nY) and cucumber virus 3 (nX NY). The antibody response in the rabbit will probably not be directly proportional to the amount of each antigen present, but the quantitative difference between the two components of the antiserum will be smaller than that between the two components of

the antigen ; for, during the immunization of the rabbit, the major antibody response will reach a maximum while the minor antibody concentration in the serum is still increasing. If we take the purely arbitrary values of 80 for N and 1 for n, then tobacco mosaic antiserum might have the structure 80 anti-X and 10 anti-Y. Similarly cucumber virus 3 would be 1X and 80 Y while its antiserum would be 10 anti-X and 80 anti-Y. If these units are taken as representing optimal combining amounts, *i. e.* if 1 unit of X combines with 1 unit of anti-X and 1 unit of Y with 1 of anti-Y, and if a precipitation test with constant antiserum is considered using both homologous and heterologous antigen, then a result of the type shown in Table IV would be expected if we assume that inhibition in the region of antigen excess takes place as in the experiments that we have already described.

TABLE IV.—*Antiserum* (*Tobacco Mosaic Virus*).

Anti-X		80	80	80	80	80	80	80
Anti-Y		10	10	10	10	10	10	10
Homologous antigen concentration (T.M.V.)	X	320	160	80	40	20	10	5
	Y	4	2	1	..	..	..	..
X-effect		+++	++++	++++	++++	+++	++	+
Y-effect		+	−	−	−	−	−	−
Combined effect		++++	++++	++++	++++	+++	++	+
Heterologous antigen (C.V. 3)	X	4	2	1	..	..	..	..
	Y	320	160	80	40	20	10	5
X-effect		−	−	−	−	−	−	−
Y-effect		−	−	−	+	++	++	+
Combined		−	−	−	+	++	++	+

It will be seen on reference to Table II that results of this type are obtained when either tobacco mosaic virus or cucumber virus 3 are titrated against their homologous and heterologous antisera, the antisera being used at a constant dilution, and that the large zones of non-precipitation obtained with the heterologous antisera can be explained on the basis that the precipitates are produced only by the minor antibody and the major antigen. An antigenic relationship of this type also explains the results of the cross-absorption experiments, for it is obvious that absorbing at the constant serum optimum with the heterologous antigen will remove all the minor antibody but only slightly reduce the major antibody, and the precipitation of the serum with its homologous antigen will be but little affected.

Although the antisera to the different viruses are demonstrably different in their precipitating antibody content, no differences have been found in their virus-neutralizing antibodies. When antisera to cucumber virus 3 or to any of the three strains of tobacco mosaic virus were mixed *in vitro* with any of the three strains of tobacco mosaic virus, they all proved equally effective in reducing the number of local lesions produced in *N. glutinosa*. The neutralizing effect is approximately proportional to the concentration of the antiserum and no " zone phenomena " have been noted.

VIRUS-CLUPEIN PRECIPITATES.

We have already shown (Bawden and Pirie, 1937*b*) that a paracrystalline precipitate, closely resembling that obtained with acid or ammonium sulphate, develops when neutral solutions of clupein and tobacco mosaic virus are mixed, and that the solubility of the precipitate in salt solution varies with the different virus strains. This phenomenon has now been investigated more fully, and certain similarities and differences in the behaviour of the three tobacco and two cucumber viruses have been determined. The precipitation appears to occur immediately the virus and clupein are mixed; it is greatly affected by changes in pH value and salt concentration, but is unaffected by small variations in temperature.

In the experiments described the amount of purified virus used was constant at 1 mg., while the other components of the system were varied and the final volume of the mixtures was 3·5 c.c. Readings of the extent of the precipitation were made by measuring the opacity of the suspensions with a photoelectric cell. When mixed with neutralized solutions of clupein sulphate (B.D.H.) none of the viruses gave a perceptible precipitate with 0·006 mg., and all gave maximum opacity with 0·05 mg. On the basis of their behaviour with intermediate quantities of clupein sulphate it was possible to divide the viruses into two groups; for tobacco mosaic and Enation mosaic viruses are more easily precipitable than Aucuba mosaic virus or either of the cucumber viruses: for example, with 0·04 mg. both tobacco mosaic and Enation mosaic viruses developed rather more than 50 p.c. of the maximum opacity, whereas the other three developed only 10 p.c.

Much more striking differences are obtained by varying the pH values and the salt contents of the mixtures, and these effects lead to a much better defined division of the viruses into two groups. When a system containing 0·06 mg. of clupein sulphate and 1 mg. of virus in 3·5 c.c. is studied, it is found that the precipitates with Enation mosaic and tobacco mosaic viruses are less soluble in the presence of salt than the precipitates with the other three viruses. For example, at pH 5·5 a suspension of the clupein compounds of Enation mosaic or tobacco mosaic virus in $M/60$ phthalate buffer has only half the opacity of a similar suspension in water, whereas with the other three viruses it is only necessary to raise the salt concentration to $M/200$ at this pH to produce this reduction in opacity. On either side of pH 5·5 the precipitates with all 5 viruses become less soluble in salt, and approximately twice the concentration is necessary to reduce the opacity to half at pH 5 or 6; thereafter the solubility rises again. The exact behaviour depends to a slight extent on the clupein-virus ratio, the strain of virus used and the particular salt; it is therefore unprofitable to attempt at present to give any great precision to a description of the system. It is clear, however, that under similar conditions the solubility minima have nearly the same pH values for all five viruses.

How far these results are generally applicable to viruses is unknown, but purified preparations of potato virus "X", which are also liquid crystalline, give with clupein sulphate an amorphous precipitate which is presumably

analogous to the para-crystalline precipitates described here. In interpreting histological appearances of virus-infected plants it is important that the existence of these complexes should be realized, for they are insoluble under conditions which may exist in the interior of cells, and similar virus precipitates are formed with some histones and protamines other than clupein.

It is well known that plants infected with strains of tobacco mosaic virus contain intracellular inclusions of two types. The one consists of rounded vacuolate bodies (X-bodies) and the other of flat plates. Iwanowski (1903) showed that the latter become striated when made acid, and Goldstein (1926) confirmed this, and stated that when acidified they seemed to be made up of distinct rods or needle crystals. Beale (1937) has pointed out that the needles obtained by acidifying these plates closely resemble the " crystals " described by Stanley (1936) in his acidified tobacco mosaic virus preparations, and suggests that the plates are the source of the virus. Examined between crossed Nicol prisms the plates are seen to be birefringent when viewed edgeways but not when viewed flat. As many of the plates are definitely hexagonal this suggests that they may be true hexagonal crystals, for these are birefringent only when viewed along the transverse axes. Highly purified preparations of all five viruses give birefringent solutions, jellies and paracrystals, but no true crystals comparable to those seen in plants infected with strains of tobacco mosaic virus have yet been prepared. If, as the evidence suggests, the hexagonal crystals are depositions containing virus, there are several possible explanations for the different behaviour in the plant and after isolation. In the plant a process of slow crystallization which has not been simulated *in vitro* may go on ; or the plates may be composed of a virus-host complex similar to the precipitates we have described with clupein ; or the different crystalline states may have their origin in the different physical states of the virus before and after isolation. We have previously produced evidence indicating that during the process of purification the virus undergoes an irreversible aggregation, and it is possible that small units as they occur in the plant can arrange themselves in true crystals, whereas the larger aggregates in the isolated virus preparations cannot. The X-bodies are apparently quite different structures from these plates, for they are not birefringent, and Beale (1937) has shown that they are unaffected by acid.

In cucumber plants infected with viruses 3 and 4 no intracellular inclusions have been seen, and if they occur it must be much more rarely than in solanaceous plants infected with the strains of tobacco mosaic virus. The source of the virus therefore cannot lie entirely in the inclusions, as Beale suggests, but the absence of crystals from infected cucumbers might lend support to the idea that their production is a function of the concentration of virus ; for, as we have stated above, the yield of cucumber viruses per volume of expressed sap is much less than that of the tobacco mosaic viruses. Other factors, however, might equally well explain the absence of inclusions : for example, the sap of cucumbers is very much more alkaline than is that of solanaceous plants, and this would increase the solubility of either the viruses or of virus complexes of the type we have described.

DISCUSSION.

It has been realized for some years that certain plant viruses occur in numerous strains: in general the strains possess similar host ranges and properties, but are differentiated because they cause different symptoms. A considerable amount of circumstantial evidence has also been accumulated, indicating that strains are continuously arising by a process analogous to mutation. Various workers have shown that recognized virus strains are closely related serologically, and that they have the power of immunizing plants against one another. A relationship of the type that exists between the tobacco mosaic viruses and cucumber viruses 3 and 4 does not seem to have been described before. Cucumber viruses 3 and 4 were recognized to be related strains, but because of their different host range they had not previously been thought to be related to tobacco mosaic virus. From our results it is apparent that the five viruses studied fall into one main group; the analytical figures for all are so similar that they afford no differentiation, and the differences in the physical and chemical properties yet found are on the whole trivial. They are sufficient, however, to show that the nucleoproteins isolated from plants infected with strains differentiated on phytopathological grounds are different proteins. They also show that the greater the differences in host range and symptoms caused, the greater the differences that can be detected in the properties of the isolated viruses, and suggest the advisability of differentiating and grouping viruses by other than usual phytopathological methods. Of these, the serological technique and X-ray analysis would appear to be most useful. Straight precipitation tests are sufficient to distinguish between the tobacco mosaic viruses and the cucumber viruses, but to distinguish between the individual strains of tobacco mosaic virus the more sensitive cross-absorption test must be used. The X-ray measurements (Bernal and Fankuchen, 1937) show differences of the same order: all five viruses pack in the same manner, indicating that they are of the same general shape, but measurements of the main spacings are sufficient to distinguish between the tobacco mosaic viruses and the cucumber viruses, the latter having a smaller cross-section. The three strains of tobacco mosaic virus all give the same main spacings, but a consideration of the relative intensities of all the lines on the X-ray plate separates each strain with certainty from the others.

The relationship of cucumber viruses 3 and 4 to the tobacco mosaic viruses is difficult to define. It was suggested (Bawden, 1934) that, in discussing the potato virus "X" group, relationships are found analogous to those indicated by genera, species and varieties. If this view be adopted here then all five viruses examined could be regarded as belonging to one genus, cucumber viruses 3 and 4 as varieties of one species and the three strains of tobacco mosaic virus as varieties of a second species of the same genus.

Most plant viruses are not serologically related to tobacco mosaic virus and possess quite different properties *in vitro*. The fact that the five viruses described form such an uniform group gives us no reason to imagine that all other plant viruses are necessarily similar in their chemical properties.

SUMMARY.

Methods are described for the isolation of nucleoproteins from cucumber plants infected with cucumber viruses 3 and 4. These have not been isolated from uninfected plants, and all the available evidence indicates that they are the viruses themselves. Infections were obtained with 10^{-10} g., and specific precipitates with antiserum with $1/8 \times 10^{-6}$ g. Concentrated solutions are spontaneously birefringent and dilute solutions show anisotropy of flow: when sedimented by high-speed centrifugation they form birefringent jellies, and when precipitated with acid or ammonium sulphate they form needle-shaped para-crystals. Although these viruses have a distinct host range from tobacco mosaic virus, the purified preparations have similar chemical compositions and many properties in common with purified preparations of strains of tobacco mosaic virus; they differ from tobacco mosaic virus, however, more widely than the recognized strains of tobacco mosaic virus differ from each other. The cucumber viruses and the tobacco mosaic viruses have common antigens: the results of cross-absorption experiments between the various viruses and their antisera are described, and provisional antigenic formulæ suggested. Possible methods of relating and distinguishing between viruses and the relationship between the cucumber and tobacco viruses are discussed.

We have great pleasure in thanking Dr. G. C. Ainsworth for supplying us with cucumber viruses 3 and 4, Mr. E. T. C. Spooner for preparing the antisera used, and Prof. A. A. Miles for suggesting the quantitative interpretation of our serological results.

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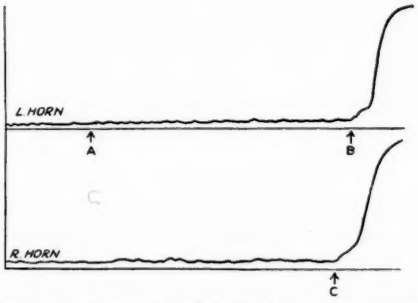
A NOTE ON ANAPHYLAXIS WITH TOBACCO MOSAIC VIRUS PREPARATIONS.

Chester (1936) showed that normal tobacco protein is strongly anaphylactogenic but that tobacco mosaic virus is not, for guinea-pigs sensitized with sap from infected plants could be desensitized completely with the sap

from healthy plants of the same species. Chester tested several preparations of tobacco mosaic virus prepared by Stanley, and although he found that they were not active anaphylactogens, yet they all reacted with guinea-pigs sensitized with healthy tobacco sap. Because of this Chester stated that the preparations of tobacco mosaic virus then available (1936) contained demonstrable amounts of normal plant proteins.

Dr. K. S. Chester has kindly tested one of our highly purified spontaneously birefringent solutions of tobacco mosaic virus which had been subjected in the course of the preparation to treatment with trypsin (Bawden and Pirie, 1937). This is the only preparation in which he has been unable to demonstrate the presence of normal protein by the anaphylactic test.

Thirteen pigs were injected by Dr. Chester with doses varying from 0·1 to 10·0 mg. of our preparation. The uterine horns from these pigs gave no



Kymograph tracing with the two horns of the uterus of a guinea-pig sensitized with 1 mg. of normal tobacco protein 19 days before. At A, 10 mg. of our preparation was added, at B 1·5 mg. of normal tobacco protein, and at C, 5 mg. of a preparation made by Stanley's technique.

reaction with normal tobacco protein or with tobacco mosaic virus preparations made by us or in the U.S.A. by Stanley's technique.

Six guinea-pigs sensitized with 1 mg. of normal tobacco protein reacted strongly both with tobacco protein (1·5 mg.) and with tobacco mosaic virus (5 mg.) preparations made by Stanley's methods, involving repeated "recrystallizations" and ultracentrifuging twice, but they gave no reaction with 10 mg. of our product. The results of one of these tests are shown in the figure.

We have previously suggested that the needles precipitated from solutions of tobacco mosaic virus by means of acid or ammonium sulphate are not true crystals, and that there is no reason to assume that preparations are necessarily pure because their properties are unaffected by repeated "recrystallization". Dr. Chester's results show quite clearly that incubation with trypsin readily effects a fractionation that cannot always be obtained by precipitation methods or by centrifugation.

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THE NUTRITION OF *STAPHYLOCOCCUS AUREUS*: SULPHUR REQUIREMENTS.

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STUDY of the fundamental nutrients required by parasitic or exacting bacteria has always shown that an organic sulphur compound is necessary for growth. For instance, cystine was found indispensable for *C. diphtheriæ* (Braun and Hofmeier, 1927), whilst for another strain of this organism both cystine and methionine were necessary (Mueller, Klise, Porter and Graybiel, 1933). Cystine, without methionine, exerted a " considerable influence " in the growth of *Bact. typhosum* (Fildes, Gladstone and Knight, 1933). Both cystine and methionine were " highly important and probably indispensable " among amino-acids required by *Cl. sporogenes*, whilst mercaptoacetic acid* was a further important addition for this organism (Fildes and Richardson, 1935). Recently the growth of *Staphylococcus aureus* upon a medium containing methionine and sodium dithiodiacetate,† but no cystine, has been demonstrated (Fildes, Richardson, Knight and Gladstone, 1936). The present paper describes a further study of the sulphur requirements of this organism with reference (*a*) to the compounds mentioned above, (*b*) to other compounds containing mercapto- or dithio-groups, and (*c*) to other sulphur compounds.

When the full amino-acid mixture described by Fildes *et al.* (1936) is deprived of all organic sulphur compounds it will no longer support the growth of *Staphylococcus aureus*. The addition of any one of certain compounds (for instance, cystine, methionine, mercaptoacetic acid, or certain other mercapto-compounds) will, however, permit growth. Of these compounds, cystine is

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† In lecturing to the Chemical Society on modern chemical nomenclature, Smith (1936) comments : " The nomenclature of organic compounds containing sulphur is in a chaotic state " (p. 1072). We have adopted the following rules based on Dr. Smith's recommendations :

The -SH group (hitherto described by such names as sulphydryl, thiol, thio-, -ol, mercaptan) will be denoted by the prefix mercapto- or the suffix -thiol, affixed to the name of the unsubstituted molecule (this corresponds with the use of the terms hydroxy- or -ol, respectively, for the -OH group). The corresponding -SS- compounds resulting from mild oxidation (often termed disulphides) will be denoted by the prefix dithiodi- similarly affixed. Thus, thiolacetic acid (resp. thioglycollic acid) will be called mercaptoacetic acid, and dithiodiglycollic acid will be called dithiodiacetic acid. Where compounds are referred to in the text as acids, it is understood that for test these were neutralized by addition of an equivalent amount of NaOH.

outstandingly active, and quantities as low as 5×10^{-8} *N* (which far exceeds the sensitivity of the nitroprusside test) suffice to give a positive result.

It was therefore possible that growth with added methionine, for instance, might be due to the combined influence of methionine *plus* traces of cystine contaminating the other amino-acids, and present in amounts too small to give visible growth in the control. Indeed, in experiments with the full amino-acid mixture, but lacking organic sulphur compounds, control tubes were not wholly devoid of growth, even though adjudged " negative ". The expression " negative " means in this paper only that under standard conditions of lighting there was no opacity visible to the unaided eye ; growth was always detectable on microscopic examination if natural amino-acids were present. Hence the finding that a control is " negative " in these cases does not necessarily exclude the presence of minute traces of cystine. Vickery and Leavenworth (1929) have already discussed the occurrence of cystine as a common impurity in histidine, and tyrosine also may require purification (Winterstein, 1901). Similarly there may be sufficient cystine in glutamic acid as usually prepared (King, 1932) to give a nitroprusside-cyanide test. Suspected contamination of this type leads to uncertainties in assessing the effects of cystine such as have already been mentioned (Fildes, 1935 ; Fildes and Richardson, 1935).

An attempt to ascertain the absolute effect of cystine, methionine, or mercaptoacetic acid has therefore been made in the following ways : (*a*) by careful purification of all natural amino-acids used, and by reducing the number and concentration of these as far as was possible without seriously reducing the nutrient value of the medium ; and (*b*) by omitting all natural amino-acids and using a strain of *Staphylococcus aureus* which had been trained to grow in their absence with a suitable form of sulphur (Gladstone, 1937*b*). By this latter procedure it was found that the " negative " control now exhibited no growth detectable by the microscope.

TECHNICAL.

Basal Media.

For reasons just given it would have been desirable to use a complete range of synthetic* compounds for this work. Since this was not possible to us we approximated to that ideal by modifying the basal medium of Fildes *et al.* (1936) as follows :

(a) *Use of synthetic growth factors.*—" Staphylococcus factor " was replaced in the medium by a mixture of 10^{-5} *M* s-nicotinamide and 10^{-7} *M* s-aneurin (vitamin B_1) in accordance with the findings of Knight (1937*a*, 1937*b*). " Factor III " for anaërobic growth was replaced by 5×10^{-5} *M* s-uracil (Richardson, 1936).

(b) *Use of different amino-acid mixtures.*—Two amino-acid mixtures were used :

1. *The restricted amino-acid mixture* (*Exp.* 1), omitting from the mixture of Fildes *et al.* (1936) the s-methionine and all amino-acids of natural origin

* Throughout this paper the prefix s- denotes a substance of synthetic origin.

unless necessary to maintain the nutrient value of the medium. Thus s-methionine, l-oxyproline, and d-glutamic acid were omitted and the concentrations of l-tyrosine, d-arginine and l-histidine were reduced from $M/4000$ to $M/10{,}000$. l-proline remained at $M/1500$.

2. *The s-amino-acid mixture* (*Exp.* 2), omitting the remaining natural amino-acids but including $M/1500$ s-*iso*leucine.

It will be noted that these media are composed solely of known chemical compounds.

Materials.

l-Cystine.—This was prepared from hydrolysed hair in the usual way and repeatedly precipitated by neutralizing a solution in dilute acid.

s- and l-Methionine.—s-Methionine, supplied by Messrs. Hofmann la Roche, Ltd., was found to give a distinct nitroprusside test in ammonia or bicarbonate solution, after treatment with cyanide or aluminium amalgam. Other specimens kindly given by Prof. G. Barger and Prof. C. S. Marvel gave similar tests, persisting even after resolution of the racemic mixture (we thank Prof. Marvel for this valuable specimen). On the other hand, l-methionine (for which we wish to thank Dr. N. W. Pirie) gave a less marked test, which was no longer given after four recrystallizations. It may be supposed therefore that s-methionine contains small amounts of homocystine produced probably in the final (hydrolytic) stage of synthesis. It is found that homocysteine thiolactone gives no nitroprusside test in bicarbonate-carbonate or cyanide solution, but an immediate test in ammonia solution. Fortunately, homocystine has no outstanding influence on growth (Table I), and in accordance with this result we also find that s-methionine and recrystallized l-methionine have indistinguishable effects on growth. In this work we have therefore used s-methionine without further treatment.

s-Homocystine, s-homocysteine and s-pentocystine.—These compounds, together with the thiolactone hydrochloride of s-homocysteine, were generously supplied by Prof. V. du Vigneaud.

Other amino-acids.—The *synthetic amino-acids* were obtained from Messrs. Hofmann la Roche and from the Chemistry Department of the University of Illinois. *d-Arginine* and *l-proline* were prepared from hydrolysed gelatin by the admirably specific methods of Brand and Sandberg (1932) and of Bergmann (1935) respectively. *l-Tyrosine* was a commercial preparation four times precipitated by neutralizin ga solution in dilute acid. *l-Histidine* was a commercial preparation, treated to oxidize cystine by careful addition of bromine water till a faint permanent colour remained, and then recrystallized three times from aqueous alcohol.

Each of these amino-acids was adjudged free from cystine to the limits of the nitroprusside-cyanide test.

Dithio- and mercapto-compounds.—Most of the mercapto-compounds included in Table I were prepared for test by reduction of $N/10$ solutions of the corresponding dithio-compounds. This was done for two reasons—(*a*) to compare the effects on growth of dithio- and of mercapto-compounds of identical origin, and (*b*) for convenient purification, since the dithio-compounds are

somewhat insoluble, crystallizable solids, whereas the mercapto-compounds are often highly-soluble, autoxidizable oils. Two exceptions to this procedure were homocysteine (which gives a thiolactone in acid solution) and *α*-mercapto-*iso*butyric acid (which is very resistant to reduction by Sn and HCl); these were each solids from which solutions were made up just before test. Solutions prepared by reduction had the same effect on growth as solutions made directly from the mercapto-compounds, when tested in the cases of cysteine or mercaptoacetic acid.

Mercaptoacetic acid was a commercial specimen. *Dithiodiacetic* acid was prepared from mercaptoacetic acid by oxidation in aqueous solution with H_2O_2 and $CuSO_4$ catalyst (Pirie, 1931), extraction into ether, evaporation of the dried ether solution in presence of a trace of HCl, and crystallization from ether-benzene. Other *dithio-acids* were prepared with H_2O_2+$CuSO_4$ from the corresponding mercapto-acids, which were prepared according to methods in the literature (*cf.* Biilmann, 1905, 1906) from the bromo-acids by way of the ethylxantho-acids. The mercapto-acids were not usually isolated and purified, but the ethylxantho-acids were recrystallized from benzene+petroleum ether, and the dithio-acids from ether+benzene, aqueous alcohol, or water. *Dithiodi-β-ethylamine hydrochloride* was similarly prepared by way of β-mercapto-ethylamine hydrochloride (Gabriel, 1891) from β-mercaptoethylphthalimide (recrystallized from benzene+petroleum ether), prepared from β-bromoethyl-phthalimide (Salzberg and Supniewski, 1932). The recrystallized dithio-compounds contained no free -SH group detectable by nitroprusside test.

Other sulphur compounds.—Thiourethane (*ethylxanthamide*) occurs as a bye-product of the synthesis of mercapto-acids above, and was freed from -SH by washing a solution in ether with dilute sodium carbonate, and recrystal-lizing from benzene+petroleum ether. *Ethyl-ethylxanthate* was prepared from diethyl sulphate and potassium ethylxanthate in the usual way and fractionally distilled. *α-Ethylxanthopropionic* acid was an intermediate in the preparation of dithiodi-*α*-propionic acid. *2-Thiothymine* was an intermediate in the preparation of thymine (*cf.* Richardson, 1936), *2-mercaptohistidine* was a synthetic preparation kindly given by Prof. C. R. Harington. *Cysteic acid* was a preparation from cystine kindly given by Mr. W. Lawson. Other sulphur compounds were of commercial origin.

Preparation of Test Solutions.

For Exps. I, II and III stock solutions of mercaptoacetic acid were made $M/10$ in MHCl, autoclaved, and stored in a refrigerator for not more than 6 days; for use they were diluted with sterile water to $M/100$. Cystine was prepared as an $N/100$* solution in $M/10$ HCl and methionine as an $M/20$ (or $M/100$) solution in water, and these solutions were sterilized by filtration.

If the cystine or methionine was sterilized in an autoclave they became inhibitory. The effect was more apparent with cystine, which became increas-ingly inhibitory at concentrations above $0{\cdot}04 \times 10^{-3}$ N, so that 10^{-3} N cystine

* The concentration of dithio-compounds is expressed throughout the paper as normality with respect to sulphur; thus $M/200$ cystine = $N/100$ S.

might delay growth for a number of days. In the inhibitory samples no H_2O_2 or intermediate oxidation products of cystine (Lavine, 1936) could be detected. Moreover, neither cysteic acid nor the intermediate oxidation products prepared by the method of Simonsen (1933) were inhibitory. Thus, having no explanation for the inhibition, we have simply avoided it by sterilizing the organic sulphur compounds (except mercaptoacetic acid) by filtration through a glass Seitz filter (Knight and Fildes, 1936).

Of the dithio-compounds tested in Table I, cystine, homocystine, pentocystine and dithiodi-β-ethylamine were made up $N/100$ in $M/10$ HCl, while the dithiodi-butyric and β-propionic acids were $N/20$ solutions of the sodium salts, and the remaining dithio-acids $N/20$ solutions in $M/10$ HCl. Of the mercapto-compounds, homocysteine, homocysteine thiolactone, and α-mercapto*iso*butyric acid were made up $M/100$ in $M/10$ HCl. Other mercapto-compounds were made by reduction of the dithio-compounds. This was done by heating 10 ml. of an $N/10$ solution (or suspension) of the dithio-compound in MHCl with 2 g. granulated tin in a boiling-water bath. At intervals, 1 ml. aliquots were removed, made to 10 ml., treated with H_2S, then CO_2 for 20 min., then filtered and duplicate portions titrated for -SH (Lavine, 1935). When reduction was complete (60 to 90 min.), a 2 ml. aliquot was treated in a similar way and the resulting solution of $M/100$ mercapto-acid sterilized by filtration. These solutions contained 99 to 104 p.c. of the theoretical -SH.

Technique of Test.

This followed the general procedure of Fildes *et al.* (1936), the same strain of the organism being used throughout.

RESULTS.

Effects of Cystine, Methionine, or Mercaptoacetic Acid as Sole Source of Organic Sulphur.

Early work with the full amino-acid medium (Fildes *et al.*, 1936), but lacking organic S compounds, illustrated the very great difference in activity of the above compounds as judged by the minimum effective concentration at which they permitted growth. Cystine was still effective at 5×10^{-8} N, but with methionine or mercaptoacetic acid 10^{-3} M or 10^{-5} M solutions, respectively, were required. It was desirable, as already discussed, to confirm these differences upon media less liable to contamination by cystine. This has been done in Exps. 1 and 2. With the restricted amino-acid medium (Exp. 1) the rapidity of growth was lessened and the titres of the three compounds were reduced. On the s-amino-acid medium (Exp. 2) a "normal" staphylococcus would not grow at all, and we therefore used a strain which was derived from our culture by a process of training and which would grow without addition of any amino-acid except cystine (Gladstone, 1937*b*). Nevertheless, even with this strain the rapidity of growth was again lessened, and the titres of the compounds further reduced.

EXPERIMENT 1.—*The effect of cystine, methionine, or mercaptoacetic acid in the restricted medium.*

The final medium was prepared as follows:

Restricted amino-acid mixture, autoclaved in 6 ml. lots (20 min. 115°). Constant addition per tube for aërobic growth, separately sterilized, mixed and added aseptically:

	Ml. per tube.	Concentration in final medium.
Water	1·5	—
M/500 ferrous ammonium sulphate in *M*/50 HCl (autoclaved)	0·25	$5 \times 10^{-5} M$
M/2000 s-vitamin B_1 in *M*/1000 HCl (autoclaved 10 min., 100°), diluted 1 : 100	0·2	10^{-7} *M*.
M/2000 s-nicotinamide (autoclaved)	0·2	10^{-5} *M*.
M/5 sodium nitrate (autoclaved)	0·1	*M*/500.
M/2 glucose (filtered)	0·25	*M*/80.
	2·5	

Organic sulphur compounds added in amounts shown, and volumes brought to 10 ml. with water and *M*/5 NaOH as required. Incubated at 37° in air + 5 p.c. CO_2.

Sulphur compound.	Concentration in final medium.	Days. 1.	2.	3.	4.	5.
None		0	0	0	0	0
Cystine	$40{\cdot}0 \times 10^{-6}$ *N*	0	+++	+++	+++	+++
„	$8{\cdot}0 \times$ „	0	++	++	+++	+++
„	$1{\cdot}6 \times$ „	0	+	+	+	++
„	$0{\cdot}3 \times$ „	0	tr.	tr.	tr.	+
Methionine	$5{\cdot}0 \times 10^{-3}$ *M*	0	+++	+++	+++	+++
„	$1{\cdot}0 \times$ „	0	++	++	++	++
„	$0{\cdot}2 \times$ „	0	0	0	?	tr.
Mercaptoacetic acid	$1{\cdot}0 \times$ „	0	+	++	++	++
„ „	$0{\cdot}2 \times$ „	0	tr.	+	+	+

+++ = maximum growth; other plus signs roughly proportional.
tr. = just visible opacity.
? = dubious opacity in comparison with—
0 = no visible opacity.

To decide whether the growth without cystine was actually dependent on cystine embodied in the inoculum, the growths upon 5×10^{-3} *M* methionine and 10^{-3} *M* mercaptoacetic acid were subcultured (2 drops) on the second day into the same mixture and successfully propagated for 4 serial subcultures.

EXPERIMENT 2.—*The effect of cystine, methionine, or mercaptoacetic acid in the medium containing only s-amino-acids.*

The medium was prepared from the s-amino-acid mixture and made up as in Exp. 1. The constant addition was modified to include 0·1 ml. *M*/60 magnesium sulphate (= *M*/6000 final), and 0·25 ml. *M*/7·5 ammonium sulphate (= *M*/300, to equalize the diminution in amino-N); the sodium nitrate was omitted and water added to make 2·5 ml. final volume. The inoculum was prepared from a growth on cystine-ammonium sulphate (Gladstone, 1937*b*), centrifuged, and washed twice with distilled water. Tubes incubated at 37° in air + 5 p.c. CO_2.

Sulphur compound.	Concentration in final medium.	Days. 1.	2.	3.	4.
None	..	0	0	0	0
Cystine	100×10^{-5} *N.*	0	++	+++	+++
„	$10 \times$ „	0	+	++	++
„	$1 \times$ „	0	?	tr.	tr.
Methionine	1×10^{-3} *M.*	0	?	tr.	tr.
Mercaptoacetic acid	$1 \times$ „	0	+	++	++
„	$0{\cdot}2 \times$ „	0	0	tr.	tr.

The growths upon 10^{-3} *M* methionine and mercaptoacetic acid were propagated, as in Exp. 1, through 5 subcultures upon *M*/150 methionine or 10^{-3} *M* mercaptoacetic acid respectively.

From the results in Exp. 2, in which a washed inoculum grew in serial subculture on a medium which was certainly free from unrecognized organic sulphur, it is obvious that neither cystine nor methionine are indispensable to growth of the modified strain (Gladstone) of the organism. Since the same experimental result, namely, growth in serial subculture without added cystine, followed when the normal parent strain was used on a medium which could contain only infinitesimal amounts of cystine, we see no reason to suppose that this growth on an amino-acid medium lacking cystine was dependent on undisclosed traces of cystine therein. Indeed, we conclude that normal staphylococci can dispense with cystine and methionine without serious difficulty in an otherwise adequate medium. Nevertheless, the efficiency of cystine in permitting growth, as judged by the minimum concentration required, is far superior to that of methionine or mercaptoacetic acid.

The relationship between the requirements for cystine, methionine, or mercaptoacetic acid was found to be of the same order when tested with six other representative strains of *Staphylococcus aureus*.

The Effect of Methionine on Growth with Cystine.

Although methionine as sole source of sulphur is not readily available to the organism, as a supplement to cystine it greatly accelerates the eventual growth. This was observed first in work on the full amino-acid medium, lacking organic S compounds (Fildes *et al.*, 1936), in which concentrations as low as 2×10^{-5} *M* methionine (too low to support growth alone) showed this effect. The acceleration is exhibited also on the restricted amino-acid medium, as shown in Exp. 3. It is not due to a mere summation of available sulphur, since a mixture of 2×10^{-5} *N* cystine with 4×10^{-5} *M* methionine ($= 6 \times 10^{-5}$ *N* S) has given good growth in 27 hours, whereas 20×10^{-5} *N* cystine or 100×10^{-5} *M* methionine are still without visible growth. Indeed, the effect of added methionine seems to be solely one of acceleration, since in another experiment with serial dilutions of cystine, the end-point of cystine titration was the same with or without 2×10^{-5} *M* added methionine, but was visible more quickly in its presence. Apparently, the organism suffers only slight difficulty, represented by some hours' delay in growth, in synthesizing from cystine as much methionine (or metabolic derivative therefrom) as it requires; it seems, though, that any conversion of cystine in this way is smaller than the experimental error of the opacity reading. It may be mentioned that homocysteine functions as methionine in this connection, but that mercaptoacetic acid or homocystine are not made use of quickly enough to give an effect.

EXPERIMENT 3.—*The effect of methionine on growth with cystine. Details as in Exp.* 1.

Sulphur compounds (final concentrations).	Hours.			Days.		
	19.	23.	27.	2.	3.	4.
None	0	0	0	0	0	0
Cystine 20 × 10^{-5} *N* + methionine 100 × 10^{-5} *M* .	tr.	++	+++	+++	+++	+++
„ „ × „ + „ 20 × „ .	0	tr.	+++	+++	+++	+++
„ „ × „ + „ 4 × „ .	0	?	++	+++	+++	+++
„ „ × „ + „ 0·8 × „ .	0	?	++	+++	+++	+++
„ „ × „	0	0	0	+++	+++	+++
„ 2 × „ + „ 100 × „ .	0	tr.	++	+++	+++	+++
„ „ × „ + „ 20 × „ .	0	?	++	+++	+++	+++
„ „ × „ + „ 4 × „ .	0	0	++	+++	+++	+++
„ „ × „ + „ 0·8 × „ .	0	0	0	+++	+++	+++
„ „ × „	0	0	0	tr.	++	+++
„ 100 × „ .	0	0	0	.	tr.	tr.
„ 20 × „ .	0	0	0	.	tr.	tr.
„ 4 × „ .	0	0	0	.	0	?
„ 0·8 × „ .	0	0	0	.	0	0

Effects of Other Mercapto- and Dithio-Compounds.

The compounds tested so far have had the common property that they contained actually or potentially a mercapto-group. It was decided therefore to test other mercapto-compounds which could be directly derived from them, *e. g.* homocysteine from methionine by demethylation, and β-mercapto-propionic acid or β-mercaptoethylamine from cystine by deamination or by decarboxylation. Since such tests revealed considerable qualitative differences according to structure, a further series of mercapto-compounds was studied.

It is common knowledge that mercapto-compounds readily undergo atmospheric oxidation, especially at neutral or alkaline pH in presence of iron as in this medium ; moreover, the rate of autoxidation is considerably influenced by modifications of structure (Fruton and Clarke, 1934). Thus it was possible that the differing activities of the mercapto-compounds might be related to the rate at which the mercapto-group was removed from effective action by oxidation. On the other hand, dithio-compounds undergo hydrolytic decomposition in neutral or alkaline solution with production of mercapto-compounds as one of the products (the dithiodiacetate used by Fildes *et al.* (1936) contained distinct amounts of mercaptoacetate). Many factors are involved in this complex reaction, but its rate is likewise influenced by structural modifications (Schöberl and Eck, 1936 ; Fruton and Clarke, 1934). It was therefore impossible to be certain in what condition mercapto- or dithio-compounds would exist in the medium after 10 to 20 hours' incubation at 37°.

Nevertheless, an attempt to discover differences in the nutritional activity of analogous mercapto- and dithio-compounds was made by preparing the compounds simultaneously as already described, and testing them in pairs under identical conditions, both aërobically and anaërobically. At the time of addition the mercapto-compounds were almost wholly in the reduced state and the dithio-compounds were entirely free from -SH detcetable by the nitroprusside test. At the end of the aërobic experiments, mercapto-compounds

added at $M/1000$ still gave strong nitroprusside tests (except cysteine and β-mercaptoethylamine), while after either aërobic or anaërobic (with added pyruvate) experiments dithio-compounds added at $M/1000$ were still free from detectable -SH (except dithiodiacetate). We consider therefore that during an experiment the major portion of a compound under test had remained unaltered in the medium. Table I shows the lowest concentration (end-titre) at which the various compounds would permit aërobic growth on the restricted amino-acid mixture, the highest concentration tested being $M/1000$. In anaërobic growth, approximately the same results were obtained except that the activities tended to be smaller. Repetition of the experiments on the full amino-acid medium of Fildes *et al.* (1936) did not reveal any important differences.

TABLE I.—*Limiting Dilutions of Mercapto- and Dithio-Compounds for Growth.*

Details as in Exp. 1. Aërobic growth after 4 days.

Mercapto-compound.		Dithio-compound.	
Cysteine	$0{\cdot}3 \times 10^{-6}$ M	Cystine	$0{\cdot}3 \times 10^{-6}$ N.
Homocysteine	1×10^{-3} M	Homocystine	$>1 \times 10^{-3}$ N.
„ thiolactone	$>1 \times$ „		
eMntocysteine	$>1 \times$ „	Pentocystine	$>1 \times$ „
β-Mercaptoethylamine HCl	$1 \times$ „	Dithiodi-β-ethylamine HCl	$1 \times$ „
β-Mercaptopropionate	$>1 \times$ „	Dithiodi-β-propionate	$>1 \times$ „
β-Mercaptobutyrate	$>1 \times$ „	Dithiodi-β-butyrate	$>1 \times$ „
Mercaptoacetate (*a*)	$0{\cdot}04 \times$ „	Dithiodiacetate (*a*)	$1 \times$ „
Mercaptosuccinate (*a*)	$0{\cdot}04 \times$ „	Dithiodisuccinate (*a*)	$1 \times$ „
a-Mercaptopropionate	$0{\cdot}04 \times$ „	Dithiodi-*a*-propionate	$(1)^* \times$ „
a-Mercapto*iso*butyrate	$1 \times$ „	Dithiodi-*a*-*iso*butyrate	$>1 \times$ „

* Dithiodi-*a*-propionate gave practically no growth at this concentration, but gave good growth at five times the concentration in the full amino-acid medium. Other compounds of even lower activity showed the same effect to a less degree.

It seems therefore that any mercapto- or dithio-compound can supply the sulphur needs of *Staphylococcus aureus* to some extent, since even the β-acids and pentocysteine gave slight growths on occasion. Cystine and cysteine are in a class by themselves and their activity is not transferred to either of the products of decarboxylation or deamination. Indeed, the only other reasonably active compounds are the *a*-substituted acids. Mercapto-compounds as a class are more active than the corresponding dithio-compounds; where exceptions to this rule apparently exist they refer to compounds (cysteine and β-mercaptoethylamine) which had become rapidly oxidized on admixture with the medium. A similar rule appears in the comparative effects of homocysteine and homocystine in accelerating growth on cystine.

Effects of Other Sulphur Compounds.

Other classes of sulphur compound were next examined, in a search for qualitative as well as quantitative differences in the availability of organic sulphur. It was known already that sulphates were inadequate for growth

since they were present in the basal medium, and we proceeded to test two intermediate classes—sulphonic acids (RSO_3H) and thionic acids (RCSOH, Smith, 1936) or rather derivatives thereof. Sulphonates contain their sulphur in the same state of oxidation as in sulphates, so that little activity was to be expected for these compounds. On the other hand, thionic acids contain bivalent sulphur, and the thionamides from them ($RCSNH_2$) may be supposed, in particular, to be potentially mercapto-compounds owing to a tautomeric equilibrium; thus $CH_3.CSNH_2$ (thioacetamide) $\rightleftharpoons CH_3.C\langle^{SH}_{NH}$ and $NH_2.CSNH_2$ (thiourea) $\rightleftharpoons NH_2.C\langle^{SH}_{NH}$. These compounds were therefore particularly interesting to test.

Tests were made aërobically on both the full amino-acid medium and the restricted amino-acid medium as in Exp. 1. The three sulphonic acids tested (cysteic acid, taurine, and 2-naphthylamine-6, 8-disulphonic acid) and three of the compounds of thionamide type (thioacetamide, thiothymine, and mercaptohistidine) were completely inactive, mercaptohistidine up to $M/250$ and the others up to $M/500$. On the other hand, the two thionamides, thiourea ($NH_2.CSNH_2$) and thiourethane ($C_2H_5O.CSNH_2$), could supply the needs of the organism at limiting dilutions of $0{\cdot}2 \times 10^{-3}$ and 1×10^{-3} M respectively. Now, as these compounds were both derivatives of thiocarbonic acid, derivatives of dithiocarbonic acid were also tested. These, ethyl ethylxanthate ($C_2H_5O.CSSC_2H_5$) (made up $M/10$ in alcohol) and α-ethylxanthopropionic acid ($M/100$ Na salt), were active at limiting concentrations of 1×10^{-3} and $0{\cdot}008 \times 10^{-3}$ M respectively. The reactivity of these compounds cannot depend on a tautomeric change; it depends rather upon a production of mercapto-groups either by hydrolysis or by reaction with ammonia or an amine (producing a thiourethane from the second S):

$$C_2H_5O.CSSC_2H_5 + H_2O = C_2H_5OH + C_2H_5SH + COS.$$
$$C_2H_5O.CSSC_2H_5 + NH_3 = C_2H_5O.CSNH_2 + C_2H_5SH.$$

In agreement with this, all solutions at the time of test were free from -SH (as judged by a nitroprusside test in carbonate-bicarbonate solution), but on standing a few hours at room temperature the α-ethylxanthopropionate had already liberated demonstrable amounts of -SH. Since in the medium the bye-products of decomposition of ethylxantho-compounds may be biologically active thiourethanes, these may account for the whole of the activity of the poorly active substances, for instance of ethyl ethylxanthate.

DISCUSSION.

The hypothesis on which we have hitherto worked rests on the assumption, proved in the case of tryptophan (Fildes and Knight, 1933; Fildes, Gladstone and Knight, 1933; Gladstone, 1937*a*), that when some natural organic compound is found to be an indispensable article of diet, its indispensability is due to a failure of the organism to synthesize it itself. This may be due either to a lack of materials for the synthesis or to an inadequate concentration of the necessary enzymes.

In the case of the sulphur compounds discussed in this paper, neither of the two natural compounds, cystine and methionine, is indispensable. Yet for what may be described as the practical purposes of nutrition, cystine is indispensable, since it differs from methionine and all other compounds tested in being effective in high dilutions. According to our working hypothesis, therefore, we should conclude that so long as the materials necessary for synthesis of cystine are present, and so long as the enzymic constitution of the organism is adequate, cystine may be omitted from the diet without growth being suspended, though there may be some delay.

For synthesis of cystine, both N and S will be required in an adequate form. Our results show that the adequate form of S is a mercapto-group (-SH) present or potentially present in the molecule. Yet since all compounds containing this group are not equally effective, we conclude that the structural position of the group in the molecule is a factor affecting its adequacy. There is evidence in our experiments, for instance, that the mercapto-group is more accessible in the α position than in the β. The adequate form of N for a normal coccus is amino-N, as is shown by Gladstone's (1937*b*) experiments considered in conjunction with ours, and it is only after a process of training, *i. e.* development of enzymes to synthesize amino-groups from simpler sources of N, that non-amino-N can be utilized. Our working hypothesis thus leads us to conclude that the activity of mercapto-compounds in the growth of *Staphylococcus aureus* is exerted through synthesis of cystine, and that they differ from one another not in the availability of their S content for a direct effect upon growth, but in its availability for synthetic purposes. This accords with the observation that the coupled sulphur atoms of the oxidized (dithio-) compounds are less available for synthesis than they are in the reduced condition. Methionine on the other hand appears to fill an additional role. It supplies the essential materials for cystine synthesis only very indifferently, but has a complementary effect on the growth for which cystine is the first requirement.

Although conclusions derived from one form of life should be applied to other forms only with caution, nevertheless " the unity in the biochemistry of all living organisms is nowadays so evident that advances made in special chapters of this science will inevitably influence our insight into the biochemical behaviour of other groups of organisms " (Kluyver, 1936). So far as the biochemical associations between bacteria and animals are concerned we may refer to recent work of Fildes and Richardson (1935), Tatum, Wood and Peterson (1936), Lwoff and Lwoff (1937), and Knight (1937*a*, *b*).

The relation of S compounds to the growth of animals is discussed in the reviews of Lewis (1932, 1933, 1935), and of du Vigneaud and Dyer (1936). The belief appears to be developing that methionine can replace cystine effectively in the animal diet, and that the effectiveness of methionine is exerted through homocysteine, to which it may be converted by demethylation.

In the case of *Staphylococcus aureus*, methionine and homocysteine are each also able to replace cystine in the diet, but these compounds are very much less effective than they appear to be for animals. It is possible that this difference is one of technique. The usual studies on animal metabolism are concerned with comparatively minor increases in weight, whereas in a series of subcultures

of bacteria an increase of weight occurs which may be described as infinite. The resulting bacterium is an organism which must be cystine-free so far as cystine derived from the parent organisms is concerned, but the animal may well be supposed to contain at the end of the experiment some cystine which was present at the beginning. Thus the effect of feeding methionine to an animal in place of cystine may be compared more fairly with the stimulating effect of methionine when fed to a coccus together with cystine.

SUMMARY.

For growth of *Staphylococcus aureus* there must be included in the nutrients an organic sulphur compound containing actually or potentially a mercapto-group (-SH).

All mercapto- or dithio-compounds tested were active to a greater or less extent. Cystine was outstandingly active, giving visible growth at concentrations below 10^{-6} *M*.

Methionine acts only poorly on growth as a potential source of -SH, but has an accelerating influence on growth produced upon cystine.

It is argued that the effect on growth of a mercapto-compound is not exerted directly, but by virtue of its accessibility as a component in the synthesis of cystine.

Differences are noted between the utilization of methionine or homocysteine by staphylococcus and by animals as reported in the literature.

Note.

By incorporating the results of this paper and other observations, the medium of Fildes *et al.* (1936) may be improved for the growth of all strains of unadapted *Staphylococcus aureus* as follows:

(1) From the list of amino-acids omit methionine.

(2) In place of the materials sterilized and added separately the following should be used for aërobic growth:

	Ml.
Water	2·3
Ferrous ammonium sulphate, *M*/500 in *M*/50 HCl (autoclaved)	0·25
$MgSO_4.7H_2O$, *M*/60 (autoclaved)	0·1
$NaNO_3$, *M*/5 (autoclaved)	0·1
s-aneurin (vitamin B_1), *M*/2000 in *M*/1000 HCl autoclaved 10 min. at 100° C. Diluted 1/100	0·2
s-nicotinamide, *M*/200 (autoclaved) diluted 1/10	0·2
s-methionine, *M*/100 (filtered)	0·2
l-tryptophan, *M*/1000 (autoclaved)	0·1
l-cystine, *N*/100 in *M*/10 HCl (filtered)	0·2
NaOH, *M*/5 (autoclaved)	0·1
Glucose, *M*/2 (filtered)	0·25
	4·00

For anaërobic growth the following are also added, the water being reduced in proportion :

s-uracil, $M/500$ (autoclaved)	0·25
Pyruvic acid, $M/10$ (filtered)	0·15
$NaHCO_3$, $M/2$ (filtered)	0·5

For demonstrating the effect of uracil in anaërobic growth, $NaNO_3$, must be omitted.

Aërobic cultures are incubated in air + 5 p.c. CO_2 ; anaërobic in hydrogen + 5 p.c. CO_2.

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FURTHER INVESTIGATIONS ON THE CHEMISTRY, TOXICITY AND OTHER BIOLOGICAL PROPERTIES OF DIFFERENT FRACTIONS OF DYSENTERY BACTERIA.

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THIS paper is a continuation of our previous work on the toxicity and antigenic properties of *B. dysenteriæ* Shiga (Olitzki and Leibowitz, 1935). In the former paper the experiments dealt with the toxic action of the different fractions, their lethal effect on animals, and the content of skin-preparatory and injury-producing factors in the Shwartzman test. The experiments reported below relate to two other phenomena connected with the injection of bacterial antigens. The first was observed by Roemer (1891) and Kanthack (1892), and studied by Loewit (1892) and Goldscheider and Jacob (1894). They found that after the intravenous injection of a bacterial antigen there was at first a diminution of leucocytes, and 4 to 6 hours later an increase. The other phenomenon was first described by Menten and Manning (1925) and Zeckwer and Goodell (1925) and recently studied extensively by Delafield (1931, 1932, 1934). These authors showed that the injections of bacterial antigens into rabbits produced within 2 hours a hyperglycæmia and a fall in the inorganic blood-phosphorus, followed after 24 hours by a hypoglycæmia and a definite rise in the inorganic phosphorus above the initial value.

The purpose of the experiments reported in this paper was to study the relation of these reactions to each other, and to ascertain whether these different phenomena are independent or associated, and whether they are due to the same or different bacterial substances. Another related question was the behaviour of immunized animals to the action of the various bacterial substances, whether immunity towards one of these factors is connected with immunity against the other factors, and whether it is possible to produce separately immunity against the effects of each of the different substances.

TECHNIQUE.

The technique for the preparation of the different fractions of bacteria was the same as that reported in the former communication. The fractions were the P 1, P 2 and C of Furth and Landsteiner (1928) and the hapten (Ha) of White (1932, 1933). The animals received the different fractions intravenously. Three hours after the injection blood was taken from the ear vein for leucocyte counts and for blood-sugar tests. The animals were weighed during the whole period of the experiment and changes in body-weight and toxic symptoms recorded.

In our previous communication (1935) it was noted that the crude P 1 and P 2 extracts contained also carbohydrate substances. In the following experiments we used only fractions purified by repeated precipitation with acetic acid.

The bacteria used for the isolation of the carbohydrate fraction were filtered three times through cotton and washed twice with saline solution in order to remove agar particles and non-specific bacterial products, which may, according to Morgan (1936) and Pirie (1936) contaminate the specific bacterial carbohydrate. The Ha-substances were precipitated by alcohol, alsolute alcohol and ether, and dried over P_2O_5 at 78° C.

CHEMICAL CHARACTERISTICS OF THE VARIOUS FRACTIONS.

The substances from *B. dysenteriæ* Y and Flexner were white powders, while that from *B. dysenteriæ* Shiga was grey. All were easily soluble in dilute NaOH and precipitated by weak acid. They were insoluble in cold water, but swelled on heating and formed gels on cooling. Molish and Lasseigne reactions were positive, the protein reactions (trichloracetic acid, Esbach solution, ninhydrin reaction) and the Biuret test were negative.

The properties of the different substances are shown in the following table :

TABLE I.—*Properties of Carbohydrates Isolated from* B. dysenteriæ.

	Flexner. p.c.	Y. p.c.	Shiga. p.c.
Insoluble residuum after hydrolysis	4·6	4·0	10·0
Nitrogen content of the soluble material :			
1. Preparation	4·1	0·9	5·9
2. ,,	3·0	2·0	5·6
Phosphorus content	Less than 0·5	Less than 0·4	1·3
$[\alpha]_D$	+70°	+50°	+19·5°
Orcin reaction	Strongly positive	Strongly positive	Positive
Naphthoresorcin reaction	Ditto	Positive	,,
Fuchsin-sulfurous acid	Slightly pink colour	Red colour	Strong red colour
Fermentations :			
Brewer's yeast	Positive	Negative	Positive
Lactose yeast	..	Positive	..
Reducing power : p.c. of the equivalent amount of glucose	..	80	..
Mucic acid	..	Positive	..

The chemical investigations in all cases had to be made with substances less than 0·1 g. (or even 0·05 g.) in amount. This accounts for the incompleteness of the results.

It will be noted that the $[a]_D$ value of Ha(Y) excludes the presence of significant quantities of the lævo-rotary fructose a d renders the presence of the weakly dextro-rotary mannose and xylose unlikely. The presence of galactose is shown by the fermentation test and by the production of mucic acid. Nine mg. of this substance were oxidized with dilut nitric acid and the evaporated residue was triturated with 96 p.c. alcohol, washed and dried; the residue weighed 5·5 mg. On recrystallization from 67 p.c. alcohol a small quantity of crystals was received, which after drying had a melting-point of 212° C. Since according to van der Haar 5·5 mg. mucic acid corresponds to 7 mg. galactose or galacturonic acid, the Ha(Y) contains, therefore, about 77 p.c. galactose. The presence of xylose as the pentose constituent is highly improbable, because of the high rotation value of the product; it seems, therefore, that this constituent is arabinose.

Regarding the Ha (Flexner) the high $[a]_D$ shows that it contains, in addition to glucose, a large amount of l-arabinose ($[a]_D$ 105°).

The Ha(Shiga) on hydrolysis for one hour developed a yellowish-brown coloration. Even after hydrolysis with 0·2*N* HCl a yellow colour was noted. Phenylhydrazine did not cause any precipitation in the cold, but when heated a precipitate formed which could not be cleaned. With p-nitrophenylhydrazine a red precipitate was formed in the cold which, when reprecipitated from alcohol, showed an indistinct crystalline structure. This product contained 8·2 p.c. nitrogen, less than that calculated for a p-nitrophenylhydrazone or osazone of a sugar or uronic acid. The question remains whether this low value is due to an admixture of a nitrogen-free substance. A small amount of the hydrolyzed preparation was neutralized with NaOH, evaporated, and the residue extracted with 60 p.c. heated alcohol. The extract, which is free from uronic acid, contained nitrogen; when heated with p-nitrophenylhydrazine in HCl solution and repeatedly precipitated some star-like red needles were isolated, which are under investigation. It seems that the Ha(Shiga) is not identical with the substance isolated by Morgan (1936) by another method, nor could it be identified with any of the non-specific carbohydrates isolated by him.

These results show that there are striking differences in the chemical structure of the carbohydrates isolated from the three different types of dysentery bacteria. The carbohydrates belong to the group of the hemicelluloses; they swell in hot water and on cooling form agar-like gels; acid hydrolysis changed them into mixtures of reducing saccharides, and in every case the hydrolysates contained nitrogen and phosphorus. In the case of the Shiga bacterium it could be shown that the nitrogen is a constituent of the carbohydrate molecule; in the case of *B. dysenteriæ* Flexner and Y, it is possible that the carbohydrates were contaminated with small quantities of proteins. Regarding the phosphorus, the quantities present in the products of *B. dysenteriæ* Flexner and Y are so small that it can hardly be considered a constituent of the molecule. In the Shiga the phosphorus content would correspond to a molecular weight of 2500, *i. e.* one atom phosphorus to 15 sugar radicals.

There is an interesting difference between the sugars of the Flexner and Y bacteria and the more labile product of the Shiga bacterium. *B. dysenteria* Y contained galactose and galacturonic acid and a pentose, probably arabinose.

The product of *B. dysenteriæ* Flexner was similar to that of *B. dysenteriæ* Y, but it contained much more pentose and some glucose. It was more difficult to ascertain the character of the Shiga product. Together with an uronic acid, probably glucuronic acid, it contained an amino-sugar from which we could prepare a crystalline derivative. It seems, therefore, that in addition to the differences in the structure, there are certain properties common to these strains; all of them contained various quantities of hexoses, Y and Shiga contained uronic acids, and Flexner and Y bacteria contained pentoses.

THE MODE OF ACTION OF THE GLYCÆMIC FACTOR.

Experiment 1.—Three rabbits received the following treatment:

1. 1 mg. P 1 (Shiga) intravenously and 2 hours later 2 g. galactose *per os*.
2. 1 mg. P 1 (Shiga) intravenously and 24 hours later the same quantity of galactose as 1.
3. Only galactose *per os* simultaneously with 1.

During the glycæmic stage the tolerance for galactose was lowered and there was a decrease in the serum diastaste (Rabbit 1 from 108 to 41, Rabbit 2 from 136 to 98 units); however, 24 hours after the injection of P 1 the tolerance of the liver returned to the normal.

Experiment 2.—The experiment showed that the hyperglycæmia after injection of P 1 is associated with a decrease in the liver glycogen. (Untreated rabbit 1·04 g.; P 1 injected 0·24 g.) Animals fed galactose showed a rise in the glycogen (1·76 and 3·62 g.), while the blood-sugar remained normal. But if before the galactose meal (2 g.) P 1 was injected, then the liver glycogen did not rise (1·03 and 1·13 g.), but the blood-sugar rose (153 and 160 mg.). It appears, therefore, that during the glycæmic stage, after the injection of P 1, the liver does not store glycogen.

Experiment 3.—Two rabbits received 0·5 c.c. Ha(Shiga). Both developed hyperglycæmia. On the following day a second injection was given. The hyperglycæmic effect was not observed. After an interval of 5 days a third injection was given. Both again developed hyperglycæmia. This experiment shows that during the hypoglycæmic phase the animals do not respond to an injection of the hyperglycæmic factor. However, if the second injection is given after 5 days the sensitivity is normal. The failure to respond is probably due to the glycogen impoverishment of the liver.

Experiment 4.—The last experiment showed that the depressing effect of insulin (2 injections of $\frac{1}{2}$ unit) is not counteracted by the P 1. Ergotamine does not act on the blood-sugar, but given in a quantity of 0·0025 mg. with P 1, it shows an antagonism against the hyperglycæmic action of the latter. This can be explained by the paralysing action of the ergotamine on the sympathetic system, and it may be concluded that the hyperglycæmic factor causes an irritation of the liver-cells through the sympathetic.

THE MODE OF ACTION OF THE LEUCOPENIC FACTOR.

Experiment 1.—A rabbit received 1·5 c.c. of P 1 (Shiga). The number of leucocytes in the peripheral blood before the injection was 6800 per c.mm. In blood taken 2 hours later from the ear vein, the right ventricle and the

left ventricle the counts were, respectively, 4400, 3500 and 3400. In other words, the leucopenia is nearly the same in all parts of the blood-stream.

Experiment 2.—Two splenectomized rabbits, as well as two normal rabbits received an intravenous injection of 1 mg. P 1(Flexner). The following changes were observed :

	Splenectomized rabbits.		Normal rabbits.	
	Number of leucocytes.	Polynuclears p.c.	Number of leucocytes.	Polynuclears p.c.
Before injection	7,600	66	7,200	35
2 hours later	2,800	39	4,700	29
4 „	5,300	82	1,600	31
6 „	17,900	88	9,100	85
24 „	16,100	78	12,500	76

These results show that :

(1) The splenectomized animals developed a relatively higher polynucleosis.

(2) The maximal decrease of leucocytes in the splenectomized rabbits reached 2 hours after injection and was followed by hyperleucocytosis.

(3) The normal rabbits showed a more prolonged leucopenia, with a maximum after 4 hours, recovery beginning after 6 hours.

Experiment 3.—Three rabbits were killed by intravenous injection of air at different stages of the experiment, the first before the injection, the second after 2 hours, in the leucopenic stage, and the third after 24 hours in the phase of leucocytosis. The findings in the lungs in all three were nearly the same ; the larger vessels were almost free from leucocytes, while the capillaries, especially in the atelectic parts of the lungs, contained large quantities.

Experiment 4.—Three rabbits were killed by ether inhalation at the same phases as in Experiment 3. The findings were the same as in Experiment 3. It seems, therefore, that the observation of Goldscheider and Jacob made during the leucopenic phase is not specific, but is connected with the appearance of circumscribed areas of atelectasis due to the mode of killing of the animals.

Experiment 5.—The spleen and bone-marrow of the rabbits were examined at different phases of the experiment. Bone-marrow was examined in frozen sections of about 15 mm. thickness, stained with oxydase stain and counterstained with " Kern-echt-rot ". The number of positive cells in a series of 25 consecutive fields (immersion 90, ocular 5) was counted. The paraffin sections were stained with May-Gruenwald-Giemsa solution. The frozen section showed in the peripheral and central parts the following numbers of oxydase-positive cells :

	Group 1. Peripheral part.	Group 2. Peripheral part.	Central part.
Normal animals	107	179	70
Leucopenic phase	35	20	Not counted
Leucocytic phase	120	197	81

The question remains open as to whether we are dealing with absolute diminution of all cellular elements in the marrow or rather a relative diminution

of the oxydase-positive cells. Preliminary studies suggest that the first is the case.

In the leucopenic phase the spleens showed large and round follicles ; sinuses wide, with large amounts of blood, containing numerous leucocytes ; the sinuses also had large macrophages, whose cytoplasm contained numerous small blue and blackish particles of irregular size. In some instances erythrophagia was observed.

IMMUNIZATION EXPERIMENTS.

The first experiment showed that the glycæmic and the leucopenic factors were present in all three fractions (P 1, P 2, C), while the endotoxic factor, causing loss of weight and enteric symptoms, was present only in the protein fractions, more particularly in P 2. It also showed that it was possible with one or two injections to immunize completely against the glycæmic, but not against the other factors. The body-weights of the rabbits were recorded during the period of the experiment. The animals treated with the protein fractions, especially P 2, showed loss of weight after all injections, reaching 12 p.c. of the body-weight. The rabbits treated with C showed a drop of 30 g. on the day after the first injection ; on the next day the weight was normal.

A further experiment showed that with at least three injections of the protein fractions it was possible to reach a complete immunity against the glycæmic, the leucopenic and the endotoxic action of the living bacilli ; on the other hand, no such immunity resulted from immunization with C.

Experiments on the Immunizing Value of the Various Fractions of B. dysenteriæ *Shiga.*

Experiment 1.—Ten rabbits were divided into five pairs. Group 1 received intravenously P 1 ; Group 2, P 2 ; Group 3, C ; Group 4, Ha ; Group 5, no

TABLE II.—*Reaction of Rabbits to P 1 after Immunization with—*

	P 1.	P 2.	C.	Ha.	Untreated.
Blood sugar (mg. p.c.) :					
Before injection . .	100	101	83	112	119
3 hr. later . . .	111	132	89	124	180
24 hr. later . . .	115	112	101	102	105
Leucocytes (1000's per c.mm.) :					
Before injection . .	4·8	4·9	3·2	6·9	6·2
3 hr. later . . .	3·8	5·6	3·6	3·1	2·9
24 hr. later . . .	16·6	8·2	14·8	15·1	15·5
Loss of leucocytes after 3 *hours* (p.c.) . . .	21	0	0	55	53
Polynuclears (p.c.) :					
Before injection . .	25	31	35	19	10
3 hr. later . . .	61	90	60	61	17
24 hr. later . . .	69	51	65	52	58

injection. At intervals of 2–3 days each animal received 0·5, 0·8, 1·0 and 1·2 c.c. of the material. One rabbit immunized with the toxic P 2 died after the three injections with intestinal symptoms; the other survived. Five days after the last injection all the animals received 1·0 c.c. of P 1 intravenously. The results are presented in Table II.

The losses in body-weight after the injection of the relatively atoxic P 1 were not great. The experiment shows that P 1 contains large quantities of the glycæmic and leucopenic factor. The best immunizing effect against the glycæmic factor was given by P 1 and C, while the immunizing effects of P 2 and Ha were incomplete. The best immunizing effect against the leucopenic factor was given by P 2 and C. The animals treated with Ha reacted like untreated animals.

Experiment 2.—Ten rabbits were divided into five groups of 2 animals and treated as in Experiment 1. One rabbit receiving P 1 and one receiving P 2 died during the immunization period. Five days after the last injection all the animals were given 1 c.c. of P 2 intravenously. The results are given in Table III.

TABLE III.—*Reaction of Rabbits to P 2 after Immunization with—*

	P 1.	P 2.	C.	Ha.	Untreated.
Blood sugar:					
Before injection	116	99	112	104	107
3 hr. later	123	130	133	97	110
24 hr. later	127	148	79	87	111
Loss of leucocytes 3 hr. after injection (p.c.)	57	50	68	78	83
Losses in body-weight after injection (g.):					
1st day	65	60	150	195	135
2nd ,,	40	40	180	175	105
3rd ,,	50	60	210	185	85

This experiment shows, in agreement with the former ones, that there is a striking difference between the protein fractions P 1 and P 2. P 1 contains the glycæmic factor and relatively little of the leucopenic and endotoxic factors. P 2 contains little of the glycæmic factor, but potent leucopenic and endotoxic factors. The glycæmic reactions of the vaccinated as well as of the unvaccinated animals were relatively weak. The best immunization against this highly toxic fraction was obtained by treatment with the protein fractions P 1 and P 2.

Experiments on the Differentiation of the Glycæmic and Leucopenic Factors.

Experiment 1.—Two rabbits received four intravenous injections of 1 mg. of Ha at intervals of 2 to 4 days. Changes in the body-weight were not observed. Neither leucopenia nor leucocytosis resulted. The changes in leucocytes and blood-sugar 3 hours after the injections were as follows:

	1st injection.	2nd injection.	4th injection.
Drop in leucocyte count (p.c.)	0	18	0
Rise in blood sugar (mg. p.c.)	30	73	4

A subsequent injection of P 2 showed that these rabbits were not immunized against the leucopenic factor; 3 hours after the injection of P 2 they showed a drop of 81 p.c. in the leucocyte count. No glycæmic reaction was observed. The substance Ha contains, therefore, only the glycæmic factor and is completely devoid of the leucopenic and endotoxic factors.

Experiment 2.—Two rabbits received four intravenous injections of the P 2 substance extracted by boiling three successive times for one hour in 70 p.c. alcohol. The quantities injected at intervals of 3 days were: 0·5, 0·8, 1·0, 1·5 c.c. The changes in the leucocyte count and blood-sugar content after the first injection were as follows:

	Leucocyte count.	Blood sugar.
Before injection	5100	104
3 hr. later	2100	98
24 hr. later	7200	105

It seems, therefore, that this substance (P 2 extracted with 70 p.c. alcohol) contains only the leucopenic but not the glycæmic factor. Seven days after the last injection one of the rabbits received 2 c.c. of P 1, the other 2 c.c. of purified P 2. Neither showed any drop in leucocyte count, while the untreated controls showed losses of 84 and 86 p.c. The rabbit injected with P 1 showed a glycæmic reaction; blood-sugar before injection was 112; 3 hours afterwards, 159; after 24 hours, 108. Purified P 2 does not contain and does not immunize against the glycæmic factor, but contains and immunizes against the leucopenic factor.

It appears, therefore, that the glycæmic and the leucopenic factors are in separate fractions. One (Ha) contains only the glycæmic factor, and another, P 2 extracted in 70 p.c. of alcohol, contains only the leucopenic factor.

Experiments on the Different Factors in Other Strains of Dysentery Bacilli (B. dysenteriæ *Flexner and* B. dysenteriæ *Y*).

Experiment 1.—Twelve rabbits were divided into three groups and each group injected with P 1 of a different species of *B. dysenteriæ*. Each rabbit received four intravenous injections of 0·5 c.c. at intervals of 4 days. After the first injection all animals showed the following blood-sugar and leucocytic reactions:

P 1 from B. dysenteriæ *Injected.*

	Y.	Flexner.	Shiga.
Blood sugar (mg. p.c.):			
Before injection	101	111	98
3 hr. later	136	125	121
24 hr. later	89	79	95
Leucocytes (1000's per c.mm.):			
Before injection	6·9	7·9	6·4
3 hr. later	2·8	3·4	2·8
24 hr. later	18·5	9·4	8·9

During the immunization period one rabbit treated with P 1 (Flexner) and two rabbits treated with P 1(Shiga) died. After the third injection the animals were tested for the leucopenic as well as the glycæmic reaction. The reactions were negative in all cases. Six days after the last injection the rabbits were divided into two groups. One received 5 c.c. of P 1(Shiga) and the other P 1(Flexner). No hyperglycæmic reactions were observed. The leucopenic reactions were as follows :

Former Treatment with P 1 of B. dysenteriæ.

Percentage drop in leucocyte count after recent injection of—	Y.	Flexner.	Shiga.	Untreated.
P 1(Shiga)	22	36	0	92
P 1(Flexner)	59	0	76	66

This experiment shows that while the antiglycæmic immunity is not specific, the best antileucopenic immunity is produced by the P 1 of the homologous strain, although a partial immunity, manifested by a relatively lower loss of leucocytes, was apparently produced with heterologous strains.

Experiment 2.—Twelve rabbits were divided into three groups, each of which received P 2, twice extracted with 70 p.c. alcohol, of the different types of *B. dysenteriæ*. Four intravenous injections were given at 3-day intervals, each time 0·5 c.c. After the first injection all animals showed the typical leucopenic reaction, but the glycæmic reaction was negative. The P 2 of each of the types was more toxic than the corresponding P 1. During the treatment one rabbit treated with P 2(Y), three rabbits treated with P 2(Flexner), and two rabbits treated with P 2(Shiga) died. Six days after the last injection the surviving rabbits received 1·0 c.c. of P 2(Flexner) intravenously. A glycæmic reaction was not observed in any. The leucocytic reaction of the surviving rabbits is shown in the following table :

Former Treatment with P 2 of B. dysenteriæ.

Percentage drop in leucocyte count after recent injection of P 2(Flexner)	Y.	Flexner.	Shiga.	Untreated.
	30	0	50	56

This experiment shows that the endotoxic effect of the various dysentery bacilli is associated mainly with P 2. The losses of animals during the immunization were higher than in all former experiments. It is also evident that in all three types the glycæmic factor is absent and the leucopenic factor present in the purified P 2 fraction. It appears that the immunity against the leucopenic factor is specific and produced only against the homologous strain.

DISCUSSION.

The experiments reported above show that the different fractions of dysentery bacteria (Shiga, Flexner and Y) contain varying proportions of biologically active and toxic substances.

The P 1 fraction contains, in addition to small quantities of endotoxic substances, a glycæmic and a leucopenic factor. The endotoxic action of this

substance is not great; from 12 rabbits treated with P 1 of the various dysentery bacilli, only 3 succumbed to the toxic effect. On the other hand, as previously shown (Olitzki and Leibowitz, 1935), this substance immunizes against the toxic effect of the highly toxic P 2, as well as against living bacteria. Furthermore, the experiments show that P 1 immunizes both against the leucopenic and the glycæmic effect of the various fractions, as well as of living bacteria.

These results with rabbits could be confirmed with human beings. The test on one adult, as well as those on children now in progress, show that P 1 produces the glycæmic and leucopenic effects without any noticeable toxic symptoms. It seems, therefore, reasonable to assume that, being relatively atoxic, it may prove of value as an immunizing agent against dysentery infections.

The substance P 2 contains the leucopenic but only little of the glycæmic factor. The glycæmic factor could be completely removed by repeated extractions with 70 p.c. alcohol. The endotoxic effect of P 2 is, however, very great; of 12 rabbits inoculated with this fraction of different types of dysentery bacteria, 6 succumbed. It cannot be stated that the P 2 of *B. dysenteriæ* Shiga is more toxic than that derived from other strains; 3 of the rabbits died from P 2(Flexner) and only 2 from P 2(Shiga).

The C substance is mixed. It contains both leucopenic and glycæmic factors. It is non-toxic; no decrease in body-weight or death occurred after its injection. It has only a weak immunizing effect against the leucopenic factor and a somewhat stronger one against the glycæmic factor.

The Ha substance obtained according to White's (1932) technique by alkaline treatment and repeated alcohol precipitations contains only the glycæmic factor. It produces immunity only against the glycæmic factor. It is non-toxic.

All three strains of dysentery bacteria contained all the different factors, and their distribution in the different fractions was also the same.

The specificity of the immunity against these factors is quite different in each case. The most complete anti-leucopenic immunity was obtained with substances derived from the homologous strain. But partial immunity could be produced by fractions derived from heterologous strains. This unspecific effect may be due to proteins common to all strains of the dysentery group. Thus, antisera prepared with P 2 substances showed low agglutination titres without clearly defined specificity.

The non-specific character of the anti-glycæmic immunity is difficult to explain. The glycæmic factor is alkali-stable and is associated with Ha, which represents the strain-specific carbohydrate hapten. Nevertheless, the antiglycæmic immunity could be obtained against homologous as well as heterologous substances. This fact is explainable only by the supposition that the specific combining group of the carbohydrate hapten is not identical with the glycæmic factor, and that other unspecific groups common to all strains are responsible for this action. This hypothesis was supported by the chemical analysis of the carbohydrates derived from the different strains. In spite of differences in structure, all three contained hexoses, but in different quantities; pentoses were common to *B. dysenteriæ* Flexner and Y, while uronic acids were present in the products of *B. dysenteriæ* Shiga and Hiss (Y).

Concerning the mode of action of the different substances we found that the hyperglycæmic stage was associated with a lowered galactose tolerance, a lowered glycogen content of the liver, and a reduction of the diastase of the blood. The blood diastase shows a tendency to decrease if there is damage to the liver-cells. A clinical investigation recently published by Rachmilewitz (1937) showed that in liver diseases there is a fall in the blood diastase, with a gradual increase concurrent with the clinical improvement. These facts suggest that these substances cause an irritation of the liver-cells, in the same manner as adrenaline. It is not clear whether the bacterial substances act directly on the liver-cells or through irritation of the sympathetic. Two facts may be mentioned in favour of the second hypothesis: First, the lack of sensitiveness of the animals towards a second injection immediately after the hyperglycæmia, which may be explained also as due to glycogen exhaustion; second, the effect of the ergotamine.

The leucopenic effect was explained by Goldscheider and Jacob as a change of the distribution of white cells between the peripheral blood and the capillary vessels of the lung, spleen and other organs. We found that the storage of white cells in the lung was not specific since it was obtained in normal as well as splenectomized animals, whereas the leucopenia was present in the central as well as in the peripheral blood-stream. On the other hand, the examination of the bone-marrow in the leucopenic stage showed that even in this early stage of bacterial action, great changes of the cell content of the marrow occurred. This aspect of the problem is now under further investigation.

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SUMMARY.

1. In three different strains of dysentery bacteria (Shiga, Flexner and Y), the following factors were found:

A. A glycæmic factor, present in the fractions P 1, C and Ha. It is also present in unpurified P 2, but after extraction with 70 p.c. alcohol it is no longer demonstrable.

B. A leucopenic factor, present in the fractions P 1 and P 2, to a lesser extent in C, and absent in Ha.

C. An endotoxic factor, present mainly in P 2 and to a small extent in P 1, but absent in C and Ha.

2. The Ha substances of all three strains contained hexoses in different quantities; pentoses were present only in the products of *B. dysenteriæ* Flexner and Y.

3. Immunity against one of the factors is not associated with immunity against another factor; thus the antiglycæmic immunity is independent of the antileucopenic immunity.

4. The leucopenic immunity is on the whole strain-specific, although some effects were observed against heterologous strains.

5. The antiglycæmic immunity is not strain-specific.

6. The hyperglycæmic reaction is associated with lowered glycogen content of the liver, lowered galactose tolerance and lowered blood diastase.

7. The leucopenic effect was observed in normal and, with a certain modification, in splenectomized rabbits as well. It was accompanied by characteristic changes in the spleen and bone-marrow.

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THE HYPOTHERMIC FACTOR OF *B. DYSENTERIÆ* SHIGA.

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IN continuation of our previous studies on the biological activities of the different fractions of *B. dysenteriæ* Shiga (1935, 1937) we examined the effect of these fractions on the body temperature of the guinea-pig after intraperitoneal injections. The guinea-pigs used for these experiments were generally no heavier than 300 g. The body temperature was measured at intervals of ½ to 1 hour, thus a true picture was obtained of its changes during the period following the injection. In addition to the fractions used in our former experiments, prepared according to Furth and Landsteiner (1928) and White (1932), we also used fractions prepared according to the method described by Raistrick and Topley (1934).

RESULTS.

Before testing the effect of the different fractions we determined the reaction following the injection of whole bacteria. Only guinea-pigs which had a rectal temperature between 38·0° to 39·0° C. were used. A fall in temperature below 38·0° was considered a hypothermic reaction and a rise over 39·0° hyperthermic. The results obtained with whole bacteria are presented in Table I.

TABLE I.—*Hypothermic Reactions Caused by Intraperitoneal Injections of Different Quantities of* B. dysenteriæ *Shiga.*

Quantity injected (mg.).	Hypothermic reaction. Beginning after (hr.).	Duration (hr.).	Lowest temperature (°C.).
2·00	2½	24	Below 35·0
1·00	4½	18	,, 35·0
0·80	3	6	37·1
0·40	5	2½	36·5
0·20	5	2	37·5
0·10	Ineffective	..	..
0·05	,,	..	..

The table shows that 2 mg. of heat-killed bacteria caused a fall in the body temperature below 35° C.; smaller quantities caused a smaller drop, and 0·1 and 0·05 mg. were ineffective. It should be noted also that when large amounts of bacteria (1 and 2 mg.) were injected the fall in temperature occurred more promptly than when smaller quantities were given. In certain cases there was a rise in temperature immediately after the injection, followed 4 to 5 hours later by a drop below normal. Sometimes the hypothermic reaction was followed by fever, but this effect was not regularly observed.

We next tried to neutralize the hypothermic effect, which seemed to be associated with the toxic effect of the bacterial substance, by different immune sera. The results were confusing because the injection of rabbit serum, normal or immune, produced a hyperthermic reaction in guinea-pigs. In order to eliminate this counter-effect the immune serum was given 24 hours before the bacteria were injected. The results are shown in Table II.

TABLE II.—*Protecting Effect of Rabbit Immune Sera Injected into Guinea-pigs a Day Before the Injection of the Antigen.*

Temperature before injection of antigen (°C.).	Time after hyperthermic serum reaction (hr.).	Serum previously injected.	Antigen injected (mg.).	Hypothermic effect. Beginning after (hr.).	Duration (hr.).	Minimum (°C.).
38·7	19	Shiga (1 c.c.)	Shiga 0·5	½	1½	37·1
38·9	7	Flexner (1 c.c.)	,,	1	½	37·8
39·0	Untreated	..	,,	2½	5	36·1

In this experiment the bacteria were given at the end of the hyperthermic reaction caused by the serum. The temperature of the animals was at that time normal. It can be noted, therefore, that the animals previously treated with immune sera showed a smaller drop in temperature, of shorter duration, than the untreated animals. The serum appears, therefore, to have a neutralizing effect. But as the Flexner serum neutralized Shiga antigen, it remained to determine whether this effect was non-specific and due to the serum protein. Experiments were, therefore, carried out with different quantities of serum and a constant amount (1 mg.) of bacteria. The results are given in Table III.

TABLE III.—*Protective Effect of Various Rabbit Sera against the Hypothermic Effect of* B. dysenteriæ *Shiga on Guinea-pigs* (250–280 *g. weight*).

Kind of serum.	Agglutination *B. dysenteriæ* Shiga.	Quantity injected (c.c.).	Hypothermic effect of *B. dysenteriæ* Shiga. Begining after (hr.).	Duration (hr.).	Miminum (°C.).
Normal	Negative at 1 : 50	2·0	½	6½	Below 35·0
,,	,, 1 : 50	0·5	½	7½	36·5
Flexner (P 1)	Positive to 1 : 500	2·0	½	6	36·0
,,	,, 1 : 500	0·5	1½	7½	36·2
Shiga (P 1)	,, 1 : 5000	2·0	½	1½	37·1
,,	,, 1 : 5000	1·0	½	4½	Below 35·0
,,	,, 1 : 5000	0·5	½	4½	,,
Control untreated	..	..	1½	12½	,,

This experiment was repeated with larger guinea-pigs (450–500 g. weight) and the results were quite different. The hypothermic effect is much stronger in animals of 250 g. than in those weighing 450–500 g. Consequently the specificity of the serum reaction could best be observed with younger animals. As it is shown in Table III, 2 c.c. of the Shiga serum reduced the duration of the hypothermic effect of *B. dysenteriæ* Shiga from 12½ hours to 1½ hours, while 2 c.c. of the normal rabbit and of the Flexner serum reduced the time only to 6–6½ hours. To eliminate the influence of the heterologous serum the experiment was repeated with guinea-pig immune sera in place of the rabbit sera. The results are shown in Table IV.

TABLE IV.—*Protective Effect of Guinea-pig Sera against the Hypothermic Effect of* B. dysenteriæ *Shiga and Hiss* (*Y*) *on Guinea-pigs.*

Antigen injected.	Kind of serum treatment.	Quantity injected (c.c.).	Hypothermic reaction. Beginning (hr.).	Duration (hr.)	Minimum (°C.).
B. dysenteriæ Shiga	Shiga serum	2·0	½	1½	37·0
,, ,,	,,	0·5	½	1½	36·7
,, ,,	Normal serum	2·0	½	3	36·7
,, ,,	Untreated	..	4	5½	36·4
,, Hiss (Y)	Shiga serum	2·0	½	3	35·2
,, ,,	,,	0·5	½	2½	36·2
,, ,,	Normal serum	2·0	½	5½	35·1
,, ,,	Untreated	..	4	6	35·9

The results with guinea-pig sera were similar to those obtained with rabbit sera. The Shiga serum produced the best effect against the hypothermic reaction of *B. dysenteriæ* Shiga, but a distinct effect could also be obtained with the Hiss (Y) serum.

It would, however, seem that the serum effect was an antibody reaction against a substance common to various species of bacteria. To clear up this point we tried to ascertain whether this effect can be produced with bacteria other than *B. dysenteriæ* Shiga. It was found that the hypothermic factor was present in *S. paratyphi B, B. pyocyaneus, Staphylococcus aureus, B. dysenteriæ* Flexner and Hiss (Y). Immunization experiments showed that one or two injections of 1 mg. of bacterial substance were not sufficient to produce

immunity in guinea-pigs, but that after three or four injections of 1 mg. some of the animals no longer gave a hypothermic reaction; after five injections of 1 mg. all animals were immunized against this quantity of bacteria, but still reacted to an injection of double the quantity (2 mg.).

Animals which had been immunized in the manner described were given 2 mg. of bacteria from different species. The treatment with Shiga bacteria produced a certain immunity against the homologous and certain heterologous strains (*B. pyocyaneus*, *B. dysenteriæ* Hiss (Y)). No effect was observed, however, against *S. paratyphi B*. It seems, therefore, that the hypothermic substance, though common to some strains of bacteria, is not immunologically uniform in all cases. This question is now under investigation with a larger series of strains.

We now proceeded to examine what fraction of the bacteria was responsible for the hypothermic reaction. The experiments bearing on this point are presented in Tables V and VI.

TABLE V.—*The Hypothermic Effect of Different Fractions of* B. dysenteriæ *Shiga.*

Name of the fraction.	Quantity injected (mg.).	Hyperthermic reaction.			Hypothermic reaction.		
		Beginning after (hr.).	Duration (hr.).	Maximum (°C.).	Beginning after (hr.).	Duration (hr.).	Minimum (°C.).
Lip	6	1½	7	40·1	..	..	..
,,	4	1½	5	39·2	..	..	..
,,	2	2	2	39·2	..	..	..
,,	1	1	6	40·8	..	..	..
Ha	8	2½	6½	39·7	..	..	..
,,	6	1½	7	39·8	..	..	..
,,	2	1½	10½	39·8	..	..	..
,,	1	1	4	40·2	..	..	..
,,	0·5	3	2½	39·6	..	..	..
P 1	2	..	..	..	1	11	36·8
,,	1	..	..	..	1½	10	Below 35·0
,,	0·5	..	..	..	4	4½	37·1
,,	0·2	½	3½	39·9	4½	3½	37·4
,,	0·1	½	4½	39·9	..	..	..
,,	0·05	1	3	39·6	4½	1	37·8
,,	0·02	1	5½	39·9	..	..	..
P 2	2	..	..	..	2	6	36·1
,,	1	1	2½	39·4	..	..	..
,,	0·5	½	4	39·8	..	..	..
Q	2	½	2	40·1	3	8	Below 35·0
,,	1	½	10	40·3	..	..	..
T	2	½	11	41·0	..	..	..
,,	1	1	12	40·5	..	..	..
Bacteria free of Q and T	4	½	2½	39·5	3½	5	36·0
Ditto	2	1	4½	40·5	..	..	..

These experiments show that the lipoid substance obtained by alcohol extraction and the carbohydrate fraction (Ha) do not produce any hypothermic effect. The effect is associated with the protein fractions, especially the fraction P 1. If the method of Raistrick and Topley (1934) is used the water-soluble and alcohol-precipitable products of trypsin digestion are active;

TABLE VI.—*The Hypothermic Effect of Fractions of* B. dysenteriæ *Shiga Obtained after Trypsin Digestion.*

Name of the fraction.	Quantity injected (mg.).	Hyperthermic reaction. Beginning after (hr.).	Duration (hr.).	Maximum (°C.).	Hypothermic reaction. Beginning after (hr.).	Duration (hr.).	Minimum (°C.).
Residue	1	..	..	..	1½	10½	35·8
„	0·2	½	3	39·7	..	..	..
„	0·1	½	2	39·6	..	..	..
F 68	1	½	½	39·1	2	13½	Below 35·0
„	0·2	½	2½	40·3	3	1	37·6
„	0·1	..	..	..	..	..	..
F 68/U	1	½	1½	39·4	2	19	Below 35·0
„	0·2	½	3½	40·9	..	..	..
„	0·1	½	5	41·5	..	..	..
F 68/50	1	..	..	..	1½	10½	Below 35·0
„	0·1	½	2	40·2	3½	4½	37·3
„	0·05	1	2	39·6	4½	1	37·4
„	0·02	..	..	..	..	..	..
F 68/60	1	..	..	..	1½	2	Below 35·0
„	0·2	1	2	39·6	4	2	36·7
„	0·1	½	5	41·5	..	..	..
F 68/68	1	..	..	..	1½	22	Below 35·0 (died)
„	0·2	..	..	..	2½	4	36·7
„	0·1	½	3	40·4	3½	1½	37·7
„	0·05	½	2	40·4	3½	3	37·0
„	0·02	3	8½	39·5	..	..	..
F 68/94	1	1	1	39·2	3	7	36·6
„	0·1	½	4	40·2	5	1½	36·8
„	0·05	½	5½	40·6	..	..	..

especially active were the fractions F 68/68 and F 68/50, quantities of 0·1–0·05 mg. producing distinct effects.

The acid-soluble proteins Q and T showed only a weak effect. The bacteria remaining after the acid extraction were effective only in quantities of 4 mg. It seems, therefore, that the substance is unstable to acid. This supposition is confirmed by the following experiment: The active fraction F 68/68 was boiled for 45 minutes in acid, alkaline and neutral solutions and then tested on guinea-pigs. The experiment showed that the hypothermic substance is heat-stable when heated in neutral saline solution. It is partially destroyed by heating in 0·1 *N* HCl or NaOH and completely destroyed by heating in 0·2 *N* alkali or acid solutions.

It remains to examine the action of the hypothermic substance in the febrile stage of an infection. Animals infected with various organisms and with temperatures above 40° C. were injected with 1 mg. of the fraction F 68/60. The results are shown in Table VII.

This experiment shows that animals suffering from a bacterial or a virus infection and showing high temperatures are much more sensitive to the hypothermic substance than are normal animals. The same quantity which in a normal animal produced only a fall for 3½ hours reduced the temperature of infected animals to lower levels and for longer periods—in one instance 24 hours.

TABLE VII.—*Influence of the Hypothermic Factor on the Body Temperature of Infected Guinea-pigs.*

Kind of infection.	Temperature on the beginning of the experiment (°C.).	Treatment.	Beginning of the drop after (hr.).	Lowest temperature (°C.).	Return to initial temperature after (hr.).
Vaccine virus	40·5	1 mg. F 68/60	1½	Below 35·0	18½
Pasteurella	40·4	1 „ „	1½	38·4	6
Salmonella typhi murium	40·2	1 „ „	1½	Below 35·0	24
Uninfected	38·3	1 „ „	2½	37·2	3½
Vaccine virus	40·2	Untreated	..	39·6	..
Pasteurella	40·4	„	..	40·0	..
Salmonella typhi murium	40·3	„	..	39·8	..

SUMMARY.

The experiments reported above indicate that there exists in *B. dysenteriæ* Shiga, as well as in some other micro-organisms, a fraction which causes a fall in the body temperature. In certain cases this drop may be preceded and followed by hyperthermic reactions. This substance has antigenic properties, and it is possible to immunize guinea-pigs against it actively by injecting large quantities (4–5 mg.) of bacteria and passively by the previous injections of immune sera.

This factor is associated with the protein fractions of the bacteria, mainly with P 1 or, if the products of trypsin digestion are used, with F 68/68. The lipoid and carbohydrate fractions produced only a rise in temperature. The effect of P 1 and F 68/68 on the body temperature is interesting, because these substances also possess high biological activity in other directions. According to Olitzki, Leibowitz and Berman (1937) P 1 has a relatively low toxicity for rabbits, but produces in these animals both hyperglycæmia and leucopenia. Olitzki recently also found that it is relatively highly toxic for mice. The fraction F 68/68 from *Salmonella typhi murium* is, according to Raistrick and Topley (1934), a substance of high toxicity and immunizing value. Delafield (1934) found that it produces hyperglycæmia, and Delafield and Smith (1936) reported certain changes in the oxygen uptake of tissues due to it. The two substances P 1 and F 68/68 seem, therefore, to be closely related, if not identical.

It is at the moment impossible to say what is the mechanism of the hypothermic effect, but it could be stated that younger animals reacted more readily; adult uninfected animals were less sensitive, and infected animals, where the temperature regulation is disturbed, are highly sensitive.

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THE NUTRITION OF *STAPHYLOCOCCUS AUREUS*; NITROGEN REQUIREMENTS.

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In a recent paper Fildes, Richardson, Knight and Gladstone (1936) described a simple medium suitable for the growth of a typical strain of *Staphylococcus aureus*. With the exception of one unknown component (Knight, 1935), the medium was entirely composed of known chemical substances. It has since been shown (Knight, 1937*a*, *b*) that the unknown component can be replaced by nicotinic acid (or nicotinamide) *plus* aneurin (vitamin B_1). Thus *Staphylococcus aureus* can now be grown in a medium of known chemical composition. Although 14 amino-acids were present, it was suggested (Fildes *et al.*, 1936) that not all these were required for growth. No evidence was offered, however, as to which were necessary.

In the present paper the amino-acid nutrition of *Staphylococcus aureus* has been investigated with a view to determining which amino-acids are important. The method has been to observe the effect on growth of omitting each amino-acid in turn from the complete mixture. Later, using the method of Fildes, Gladstone and Knight (1933), experiments were made in "training" the organism to grow on a more simplified medium. In this way it was possible eventually to remove all the amino-acids, and to grow the organism in a medium containing ammonia as the main source of nitrogen.

TECHNIQUE.

The technical details were similar to those of Fildes *et al.* (1936).

Medium.

The medium was a modification of that of Fildes *et al.* (1936).

Briefly, it contained in its complete form the following amino-acids: glycine, alanine, valine, leucine, phenylalanine, tyrosine, methionine, proline, oxyproline, aspartic acid, glutamic acid, lysine, arginine and histidine. With the exception of methionine, which was used in a concentration of *M*/50,000, the quantities were those used by Fildes *et al.* (1936). In addition tryptophan (*M*/20,000) and cystine (*N*/5000, *i. e.* *M*/10,000) were used. Details concerning the standards of purity of most of the amino-acids are given in the papers of Fildes *et al.* (1936) and Fildes and Richardson (1937). Where used, other amino-acids were prepared with similar care. In order that the amino-acid content could be varied, each amino-acid was made up and sterilized separately in concentrated solution, so that the addition of a small volume (0·1 ml.) of each gave the final concentration as required in the completed medium. All amino-acids were dissolved in water except cystine and tyrosine; these were made up in *M*/10 HCl, the pH being corrected after their

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addition to the medium. Sterilization was effected by autoclaving at 115° C. for 20 minutes, except in the case of methionine and cystine, which, as noted by Fildes and Richardson (1937), should always be filtered. For this purpose the glass Seitz filter of Knight and Fildes (1936) was used. Sodium dithiodiacetate* (dithiodiglycollate) was omitted from the medium. When an organic source of sulphur other than cystine was required $M/1000$ sodium mercaptoacetate* (thiolacetate) was used.

In experiments made after the discovery of the nature of Knight's staphylococcal growth factor (Knight, 1937), this factor was replaced by synthetic preparations of nicotinamide (10^{-5} M) and aneurin (vitamin B_1, 10^{-7} M). In addition to the inorganic salts used by Fildes, magnesium sulphate ($M/6000$) was added.

Inoculum.

Unless otherwise stated, the inoculum was taken from a 24-hour culture on a nutrient agar slope. A suspension was made in distilled water and its opacity standardized to a given number of organisms. The number of organisms inoculated is shown in the protocols.

Strains of Staphylococci.

Twenty-six strains of staphylococci were used as shown in Table I.

TABLE I.

No.	Species.	Source.
1.	*Staphylococcus aureus*	Cellulitis (Fildes & Knight, 1933).
2.	,, ,,	Septicæmia (blood).
3.	,, ,,	N.C.T.C. No. 1389 Type I (Hine).
4.	,, ,,	N.C.T.C. No. 1393 Type II (Hine).
5.	,, ,,	N.C.T.C. No. 3093, " Wood ".
6.	,, ,,	N.C.T.C. No. 3095, " Wood 46 ".
7.	,, ,,	N.C.T.C. No. 3750, " Mrs. Fox ".
8.	,, *albus*	N.C.T.C. No. 3094, " Wood 86 ".
9.	,, *aureus*	Furuncle.
10.	,, *albus*	Dissociant from strain 2.
11.	,, *aureus*	Nose.
12.	,, ,,	Granuloma of hand.
13.	,, ,,	Folliculitis of scalp.
14.	,, ,,	Osteomyelitis.
15.	,, ,,	Abscess of nasal septum.
16.	,, ,,	Conjunctivitis.
17.	,, ,,	,,
18.	,, ,,	Bronchopneumonia (lung).
19.	,, ,,	Whitlow.
20.	,, ,,	Abscess.
21.	,, ,,	Bronchopneumonia (lung).
22.	,, ,,	Aγ (Bigger, 1933).
23.	,, ,,	Sinus of leg.
24.	,, ,,	Fistula of chest.
25.	,, ,,	Mastoid abscess.
26.	,, ,,	Infected sebaceous cyst.

* For adopted nomenclature of organic sulphur compounds see Fildes and Richardson (1937).

Strains 2, 11 to 21 and 23 to 26 were supplied by Dr. L. E. H. Whitby; strain 22 was received from Dr. J. W. Bigger.

EXPERIMENTAL.

Preliminary experiments showed that all strains except Strain 3 grew well on the complete medium with the addition of cystine and tryptophan. The exception was an atypical strain, growing slowly on laboratory media and producing a viscous ropy deposit in broth.

The Effect of Tryptophan on Growth.

Fildes and Knight (1933) showed that a strain of *Staphylococcus aureus* growing on a hydrolysate of gelatin required tryptophan for growth, but was easily trained to dispense with it. The effect of tryptophan on the growth of 26 strains growing in the complete amino-acid medium is shown in Table II.

TABLE II.—*The Effect of Tryptophan on Growth of* Staph. aureus *Growing in the Complete Amino-acid Medium. Inoculum* 3 × 10^6 *Organisms per* 10 *ml. medium.*

Number of days' incubation:	Without tryptophan.					With tryptophan.				
Strain.	1.	2.	3.	4.	6.	1.	2.	3.	4.	6.
1	+++	+++	+++	++	++	+++	+++	+++	++	++
2	+++	+++	+++	++	++	+++	+++	+++	++	++
3	0	0	0	0	0	0	0	0	0	0
4	+	+++	+++	++	++	++	+++	+++	++	++
5	tr.	++	++	++	+	+−	++	++	++	+
6	+	+++	+++	++	++	++	+++	+++	++	++
7	0	0	0	0	0	+	+++	+++	++	++
8	+	+++	+++	++	++	++	+++	+++	++	++
9	++	+++	+++	++	++	++	+++	+++	++	++
10	0	tr.	+	+	+	tr.	+	+	+	+
11	0	0	0	0	0	+++	+++	+++	++	++
12	++	+++	+++	++	++	++	+++	+++	++	++
13	+	+++	+++	++	++	++	+++	+++	++	++
14	++	+++	+++	++	++	+++	+++	+++	++	++
15	+	+++	+++	++	++	++	+++	+++	++	++
16	0	++	+++	+++	++	++	+++	+++	++	++
17	0	0	0	0	0	++	+++	+++	++	++
18	0	0	0	0	0	++	+++	+++	++	++
19	++	+++	+++	++	++	+++	+++	+++	++	++
20	+	+++	+++	++	++	++	+++	+++	++	++
21	0	+++	+++	+++	++	++	+++	+++	++	++
22	++	+++	+++	++	++	+++	+++	+++	++	++
23	++	+++	+++	++	++	++	+++	+++	++	++
24	++	+++	+++	++	++	+++	+++	+++	++	++
25	++	+++	+++	++	++	++	+++	+++	++	++
26	++	+++	+++	++	++	++	+++	+++	++	++

Note.—In this and other tables plus signs refer to the degree of growth: +++ maximum growth, ++ less than maximum, + moderate, +− less than moderate, tr. trace, and f. tr. faint trace.

Of the 25 strains able to grow in the amino-acid medium, only 4 were unable to do so in the absence of tryptophan. Another 13 grew slowly, but eventually reached maximum growth. With the remaining 8 no effect was apparent. When strains giving late growth in the absence of tryptophan were subcultured on to the same medium, growth occurred without delay as if tryptophan were present. From the work of Fildes and Knight (1933), it is justifiable to assume that all strains require tryptophan for growth. The initial differences between them were differences in synthetic ability. Strains in which the effect of tryptophan was not apparent were able to synthesize it as rapidly as they could make use of it. When once growth had occurred and they had become adapted to carry out the synthesis easily, subsequent subcultures grew without delay. It is of interest that the strain described by Fildes and Knight (1933) as requiring tryptophan (Strain 1) now grew equally well in its absence. Since it was used by them, it had been subcultured daily on a nutrient agar slope. During this time it may be assumed that it had become adapted to synthesize tryptophan for itself.

Determination of Essential Amino-acids.

In experiments designed to determine the amino-acid requirements of bacteria, Fildes and Richardson (1935) have stressed the importance of using amino-acids of synthetic origin wherever possible. In the complete medium, all amino-acids were synthetic except proline, oxyproline, glutamic acid, tyrosine, arginine, histidine, tryptophan and cystine. Two of these, oxyproline and glutamic acid, were found to have no effect on growth when cystine was present in the medium. They were therefore omitted. Alanine was also excluded as being unimportant. When strains which grew well in the absence of tryptophan were used, this amino-acid was also left out.

Table III summarizes a series of experiments designed to determine which amino-acids were necessary for growth. Each amino-acid was omitted in turn from a mixture containing 12 amino-acids and the effect on growth observed.

The amino-acids that appeared to be most necessary for rapid growth were cystine, leucine, valine, proline, glycine, aspartic acid, phenylalanine and arginine. Histidine and methionine were less important, and tyrosine (except for Strain 9) and lysine were not required. In the absence of any one of the more important acids, with the exception of cystine, most strains were able to grow eventually and even produced maximum growth. No continuous growth of any strain was obtained in the absence of cystine ; the faint trace of growth apparent in some cultures after prolonged incubation was probably due to cystine carried over in the inoculum. Fildes and Richardson (1937) have shown that extremely small quantities of cystine are required by the organism for growth. They have also shown that cystine can be replaced by methionine, but that much larger quantities are required. In the above experiments the concentration of methionine was too small to have this effect.

Certain amino-acids are more important for some strains than for others. For example, leucine was primarily required by Strain 1, phenylalanine by Strain 9, valine by Strains 13, 14, and to a lesser extent by 15, aspartic acid

TABLE III.—*Determination of Essential Amino-acids.*

Inoculum	Strain 1. 30,000 organisms.						Strain 6. 3×10^6 organisms.					
Time of growth (hours)	18.	24.	50.	72.	96.	144.	16.	24.	36.	72.	96.	144.
All 12 amino-acids	+−	+	+++	+++	++	++	tr.	++	+++	+++	+++	+++
Lacking glycine	0	0	tr.	+−	++	+++	0	tr.	++	+++	+++	+++
valine	0	0	+	++	+++	+++	0	0	0	+	++	++
leucine	0	0	0	0	0	0	0	0	++	+++	+++	+++
proline	0	0	0	+	+++	+++	0	0	0	+	++	+++
phenylalanine	0	0	+	+++	+++	++	0	0	++	+++	+++	+++
tyrosine	tr.	+	++	+++	+++	++	f. tr.	+	+++	+++	+++	+++
methionine	0	+−	++	+++	+++	++	f. tr.	+−	++	+++	+++	+++
aspartic	0	0	tr.	tr.	+	++	0	0	tr.	++	+++	+++
lysine	+−	+	+++	+++	++	++	tr.	++	+++	+++	+++	+++
arginine	0	0	+−	+	++	++	0	tr.	+	++	+++	+++
histidine	0	tr.	++	+++	+++	++	0	tr.	+	++	+++	+++
arginine + histidine	0	0	0	+−	++	++	0	0	0	tr.	+	++
cystine	0	0	0	0	f. tr.	f. tr.	0	0	0	0	0	f. tr.

Inoculum	Strain 8. 3×10^6 organisms.						Strain 9. 3×10^6 organisms.					
Time of growth (hours)	16.	24	36.	72.	96.	144.	16.	24.	36.	72.	96.	144.
All 12 amino-acids	tr.	+	++	++	+	+	+−	++	+++	+++	+++	++
Lacking glycine	0	0	0	tr.	+	++	0	0	0	tr.	tr.	tr.
valine	0	0	+	++	++	+	0	0	0	++	++	++
leucine	0	+	++	++	++	+	0	+	+++	+++	+++	++
proline	0	0	tr.	++	++	++	0	0	0	tr.	++	++
phenylalanine	0	0	+	++	++	+	0	0	0	0	0	0
tyrosine	f. tr.	+−	++	++	+	+	0	0	tr.	++	++	++
methionine	f. tr.	+−	++	++	+	+	tr.	++	+++	+++	+++	++
aspartic	0	0	tr.	++	++	++	tr.	++	+++	+++	+++	++
lysine	tr.	+	++	++	+	+	+−	++	+++	+++	+++	++
arginine	0	0	+	++	++	+	0	0	tr.	++	++	+
histidine	f. tr.	tr.	+−	++	++	+	tr.	++	+++	+++	+++	++
arginine + histidine	0	0	0	0	tr.	+	0	0	+−	+	+	+
cystine	0	0	f. tr.	f. tr.	f. tr.	f. tr.	0	0	f. tr.	f. tr.	f. tr.	f. tr.

Inoculum	Strain 13. 30,000 organisms.						Strain 14. 30,000 organisms.					
Time of growth (hours)	18.	24.	50.	72.	96.	144.	18.	24.	50.	72.	96.	144.
All 12 amino-acids	0	tr.	+++	+++	++	++	+	+	+++	+++	++	++
Lacking glycine	0	0	0	+−	++	++	0	0	+−	+−	+	++
valine	0	0	0	0	0	0	0	0	0	0	0	0
leucine	0	0	+	+++	+++	++	0	f. tr.	++	+++	+++	++
proline	0	0	0	+	++	++	0	f. tr.	++	+++	+++	++
phenylalanine	0	0	0	+	++	++	0	0	+−	+++	+++	++
tyrosine	0	0	++	+++	+++	++	tr.	+−	+++	+++	++	++
methionine	0	0	++	+++	+++	++	tr.	+−	+++	+++	++	++
aspartic	0	0	0	0	0	0	0	tr.	++	++	+++	+++
lysine	0	tr.	+++	+++	+++	++	+	+	+++	+++	++	++
arginine	0	0	0	tr.	++	++	0	0	0	0	+	+
histidine	0	0	++	+++	+++	++	+−	+	++	+++	+++	++
arginine + histidine	0	0	0	0	tr.	+	0	0	0	0	0	0
cystine	0	0	0	0	0	0	0	0	0	0	f. tr.	f. tr.

Inoculum	Strain 15. 30,000 organisms.						Strain 22. 30,000 organisms.					
Time of growth (hours)	18.	24.	50.	72.	96.	144.	18.	24.	50.	72.	96.	144.
All 12 amino-acids	+−	+−	++	++	++	+	+−	+	+++	+++	+++	++
Lacking glycine	0	0	tr.	+−	+−	+−	0	0	tr.	tr.	tr.	+−
valine	0	0	0	0	tr.	++	0	0	0	+	++	++
leucine	0	0	+	++	++	+	0	0	+++	+++	+++	++
proline	0	0	0	+	++	++	0	0	0	0	0	f. tr.
phenylalanine	0	0	+−	++	++	+	0	0	0	+−	+++	+++
tyrosine	+−	+	++	++	++	+	tr.	+−	+++	+++	+++	++
methionine	0	tr.	++	++	++	+	0	tr.	++	+++	+++	++
aspartic	0	0	0	0	++	++	0	0	0	0	+−	+
lysine	tr.	tr.	++	++	++	+	+−	+	+++	+++	+++	++
arginine	0	0	0	tr.	++	++	0	0	tr.	++	++	+
histidine	tr.	tr.	++	++	++	+	0	tr.	++	+++	+++	++
arginine + histidine	0	0	0	0	0	0	0	0	tr.	++	++	+
cystine	0	0	0	0	0	0	0	0	0	0	0	0

by Strain 13, arginine by Strain 14, proline by Strain 22, and glycine by Strains 9 and 22. As in the case of tryptophan, the assumption is that all strains require all these amino-acids for growth. Differences between strains are not differences in the indispensability of particular amino-acids, but differences in the ability of strains to synthesize these acids for themselves. In the second part of this paper it will be seen that, by a process of training, *i. e.* subculturing into a still more simplified medium, such differences tend to become lost.

Changes in Nitrogen Utilization : " Training " to Dispense with Amino-acids.

That the nutritional requirements of bacteria can be changed by an alteration in the substrate on which they are grown has long been known. The literature has been reviewed by Knight (1936). Knight defines the term " training " as " the derivation of cultures having simpler nutrient requirements from cultures with a more complex nutrition ", and in this sense it is used in this paper.

By successive subcultures into a medium from which the amino-acids were progressively eliminated, Fildes, Gladstone and Knight (1933) were able to " train " *Bact. typhosum* to grow on a medium whose sole source of nitrogen was ammonia. This method has been used in the present paper. The steps in the training process are illustrated in Table IV.

It will be seen from Table IV that an organism which had become trained to grow in the absence of a particular amino-acid, *i. e.* to synthesize it for itself, had also become less susceptible to the effects of omitting other and unrelated amino-acids ; an increase in synthetic ability for one amino-acid resulted in an increase in synthetic ability for others. Thus a culture of Strain 22 which had become trained to dispense with alanine grew without delay when subcultured, not only in the absence of this acid, but also in the absence of valine, leucine or histidine, which had previously been important. The prolonged lag phase brought about by the absence of proline, glycine, aspartic acid or arginine was also shortened and finally abolished. In the same way, in a single subculture from a tube lacking proline, growth was obtained of Strain 1 in the absence of leucine, of Strains 13 and 14 in the absence of valine, and of Strain 13 in the absence of aspartic acid ; without proline, each of these strains grew without delay in the course of a single subculture.

Five strains, Strains 6, 13, 14, 15 and 22, were adapted to grow on an ammonia-cystine medium by the method shown in Table IV. With another strain (Strain 1), adaptation to grow on this medium was not so easy. The training process proceeded without difficulty until the organism was growing on a medium containing ammonia, leucine and cystine. Although earlier in the training, when several amino-acids were present, leucine could be omitted, the removal of leucine from the *simple* medium resulted in complete absence of growth. In connection with the nutritional adaptation of *Bact. typhosum*, previous work showed that the final removal of tryptophan could be effected more rapidly and constantly if, in the penultimate stage of training, a variety of nitrogenous substances were provided from which tryptophan could be synthesized (Gladstone, 1937). This method was used in the present instance.

TABLE IV.—*Simplification in Nitrogen Requirements: Training to Dispense with Amino-acids.* (*Strain* 22.)

Time of growth (hours) .	18.	24.	50.	72.	96.	144.
All 12 amino-acids .	+−	+	+++	+++	+++	++
Lacking glycine .	0	0	tr.	tr.	tr.	+−
valine .	0	0	0	+	++	++
leucine .	0	0	+++	+++	+++	++
proline .	0	0	0	0	0	f. tr.
phenylalanine	0	0	0	+−	+++	+++
tyrosine .	tr.	+−	+++	+++	+++	++
methionine .	0	tr.	++	+++	+++	++
aspartic acid	0	0	0	0	+−	+
lysine . .	+−	+	+++	+++	+++	++
arginine .	0	0	tr.	++	++	+
histidine .	0	tr.	++	+++	+++	++
cystine .	0	0	0	0	0	0

Agar → (*) →

	19.	36.	72.	120.
All 9 amino-acids .	+−	+++	+++	++
Lacking glycine .	0	+	+++	++ →
valine .	tr.	++	++	++
leucine .	+−	+++	+++	++
proline .	0	0	+	++
phenylalanine	+−	+++	+++	++
aspartic acid	0	0	0	++
arginine .	0	++	++	++
histidine .	+−	+++	+++	++
cystine .	0	0	0	0

Time of growth (hours) . .	17½.	23.	42.	72.	120.
All 6 amino-acids . .	tr.	+	+++	+++	++
Lacking glycine . .	tr.	+−	+++	+++	++
valine . .	tr.	+−	+++	+++	++
proline . .	f. tr.	tr.	+	+++	++
aspartic acid .	0	0	0	+	++
arginine . .	tr.	+−	++	++	++
cystine . .	0	0	0	0	0

	23.	57.	72.	96.
3 amino-acids . .	tr.	++	++	++
Lacking aspartic acid	f. tr.	+	+	++ →
arginine .	tr.	++	++	+
cystine .	0	0	0	0

Time of growth (hours) .	17.	24.	32.	45.	72.	84.	96.	120.
2 amino-acids . .	0	tr.	+	++	++	++	+	+
Lacking aspartic acid .	0	0	0	tr.	+	+	+	+ →
cystine . .	0	0	0	0	0	0	0	0

	16.	23.	41.
1 amino-acid (cystine) (main source of nitrogen = ammonia) . .	0	tr.	+

* The arrows represent subcultures; in each series the inoculum was taken from the culture directly in front of the arrow. It was centrifuged and the organisms resuspended in distilled water. About 3×10^6 organisms were inoculated. Throughout the process of training, the nitrogen content of the medium was made equal to that of the complete amino-acid medium by the addition of ammonium sulphate.

The concentration of leucine in the ammonia-leucine-cystine medium was reduced to give minimum growth, and to this was added a number of other amino-acids (glycine, valine, proline, phenylalanine, aspartic acid and histidine). Growth occurred in 4 days. All the amino-acids other than cystine were then omitted; after a week's delay growth was obtained on the ammonia-cystine medium, and this could be subcultured indefinitely (Table V).

After 7 daily subcultures on nutrient agar, the trained organism was still capable of growing in the ammonia-cystine medium. After 12 subcultures on agar, however, the acquired property was lost.

The Removal of Cystine.

Fildes and Richardson (1937) have studied the requirements of *Staphylococcus aureus* with respect to sources of sulphur. When the organism was growing in a medium containing several amino-acids, cystine could be effectively replaced by other organic sulphur compounds, *e. g.* mercaptoacetic acid. This, however, was not the case under the present conditions, in which no amino-acids were present except cystine itself.

Training to dispense with the addition of cystine.—Strain 6, adapted to grow in an ammonia-cystine medium, was subcultured 7 times in the same medium, containing a reduced quantity of cystine (N/16,000), together with sodium mercaptoacetate (M/1000). It was then tested for growth in the absence of cystine (Table VI).

Table VI shows that *Staphylococcus aureus*, growing in the ammonia medium, can be trained to dispense with cystine. It was thus growing on a medium from which all amino-acids were excluded. The only sources of nitrogen other than ammonia present were the minute quantities of aneurin (vitamin B_1) and nicotinamide. These were synthetic preparations. The actual constituents of the medium were as follows:

KH_2PO_4	M/30
NaOH	M/37
$FeSO_4.(NH_4)_2SO_4.6H_2O$	M/20,000
$MgSO_4.7H_2O$	M/6000
$(NH_4)_2SO_4$	M/250
Glucose	M/80
Sodium mercaptoacetate	M/1000
Aneurin (vitamin B_1)	$M/1\times10^{-7}$
Nicotinamide	$M/1\times10^{-5}$

Morphological, Cultural and Biochemical Characteristics of Strains Growing on Synthetic Media.

No permanent alteration in morphological, cultural and biochemical characteristics of strains, after growing in these simple controlled media, was

TABLE V.—*Scheme showing the Training of Strain 1 to Dispense with Leucine and Strain 6 to Dispense with Cystine.*

STRAIN 1.

Medium : Full amino-acid medium	Fewer amino-acids	Ammonia + cystine	Ammonia + cystine + 6 amino-acids	Ammonia + cystine
Leucine required.	*Leucine not required.*	*Leucine required.*	*Leucine not required.*	*Leucine not required.*

STRAIN 6.

Medium : Full amino-acid medium + SH†	Ammonia + SH	Ammonia + SH
Cystine not required (Fildes and Richardson, 1937).	*Cystine required.*	*Cystine not required.*

* Dotted arrows represent training processes. † SH added as mercaptoacetic acid.

TABLE VI.—*The Elimination of Cystine from the Glucose-Ammonia-Cystine Medium.*

Time growth (hours)	. . .	20.	24.	43.	96.	120.	24.	48.	96.	22.	47.	72.
Strain 6, adapted to utilize NH_3 and grown for 7 subcultures on a medium* containing NH_3, mercaptoacetic acid (M/1000) and cystine (N/16,000) →	NH_3 + mercaptoacetic acid (M/1000)	0	0	+−	+	++						
	Ditto (M/5000)	0	0	tr.	+−	++† →	NH_3 + mercaptoacetic acid (M/5000)	0	0	tr.† →	NH_3 + mercaptoacetic acid (M/1000)	0	+−	++
	Ditto (M/25,000)	0	0	0	0	0						

Arrows represent subcultures.
* Inoculum centrifuged, washed twice with distilled water and resuspended; about 3×10^6 organisms inoculated.
† Inoculum 2 drops inoculated direct.

found. Gelatin liquefaction, fermentation, coagulase and hæmolysin tests were all positive, when the organism was transferred to the usual laboratory media, even after it had been adapted to grow on the ammonia-cystine medium. During growth in the amino-acid medium a small quantity of hæmolysin was produced and, under certain conditions, this could be considerably increased. These conditions are being investigated and will be reported in a subsequent paper. In the ammonia-cystine medium the organism produced no hæmolysin, although, as previously mentioned, it had not lost the ability to do so when transferred to a more complex medium. No coagulase was produced in any of these media. In the ammonia-cystine medium the growth of some strains tended to be somewhat granular.

DISCUSSION.

These results suggest that differences in nutritional requirements between strains of staphylococci are largely dependent on their previous nutritional conditions. Amino-acids, which initially appear to be essential, on further adaptation are no longer so. Owing to this ease of adaptation, the term " indispensable ", as applied to the amino-acid nutrition of staphylococcus, is therefore not justified. Assuming that the organism requires all these amino-acids in its metabolic processes, the removal of each apparently essential amino-acid in the training process acts as a stimulus for the production of new, or reactivation of latent, synthesizing enzymes.

As Fildes and Richardson (1937) suggest, for an organism to grow in the absence of an essential food substance it must not only possess enzymes capable of synthesizing it, but the constituents of the medium must be capable of being used by it for synthesis. To ensure success in the training process, therefore, both these requirements must be taken into consideration. With Strain 1, for example, although synthesizing enzymes for leucine could be formed in a relatively complex medium, as shown by growth in its absence, the constituents of a simpler medium were not adequate for synthesis and growth without leucine could not occur. Whether the simplified substrate could not be used by existing enzymes or whether enzymes able to synthesize leucine could not initially be formed in the simplified medium cannot yet be determined. By the addition of amino-acids which were not required for growth, conditions were provided which allowed leucine to be synthesized, and this ability persisted after the amino-acids were withdrawn (Table V).

The apparent initial " indispensability " of cystine was not due to its being required as an amino-acid, which could not be synthesized, but to its being, with the exception of an amount of methionine too small to permit growth alone, the only organic form of sulphur present. Fildes and Richardson (1937) showed that, when other adequate forms of organic sulphur together with an adequate supply of amino-acids were present, cystine could be omitted. They concluded that cystine was synthesized from amino-nitrogen and organic sulphur. With the removal of the supply of other amino-acids, however, cystine became important not only as a source of organic sulphur, but also as a source of amino nitrogen ; no growth occurred when it was replaced by mercaptoacetic acid.

With a further training to dispense with amino nitrogen, however, cystine thereupon became wholly dispensable (Table VI).

SUMMARY.

(1) Twenty-five out of 26 strains of staphylococci grew well on a medium of known chemical composition which included 16 amino-acids.

(2) Initial differences in amino-acid requirements were found to exist among these strains. The organisms were easily adapted to utilize fewer amino-acids, when such differences tended to disappear.

(3) Finally, by a process of training, strains were produced which could grow on a medium from which all amino-acids were excluded, and whose main source of nitrogen was ammonia.

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THE PATHOGENICITY TO ANIMALS OF VIRUSES ISOLATED FROM CASES OF HUMAN INFLUENZA.

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THE recent outbreak of influenza has given further opportunity for the study of the ætiology of epidemic influenza, and the investigations detailed below are to be regarded as a continuation of earlier studies. In the great pandemic of 1918–1919 most workers recorded the great difficulty of demonstrating with constancy the presence of *B. influenzæ* in the very early stages of the disease, and this organism was found only in some 50 p.c. of the cases, whilst in the later stages it reached a much higher figure (80 p.c.). Virus investigations, however, were inconclusive and gave, in most experiments, no real support to the view that a filter-passing micro-organism was present. Perhaps the most significant finding in the epidemic was that certain strains of *B. influenzæ* were capable of producing powerful exotoxins, which on intravenous injection caused hæmorrhagic lesions in the lungs. For that reason this micro-organism could not be definitely dismissed as an unimportant factor in the ætiology of influenza (McIntosh, 1922).

A further important step towards the solution of the ætiological problem of influenza was the discovery by Shope (1931) that the disease of pigs known as swine influenza was apparently due to the interaction of two ætiological agents—*B. influenzæ suis* and a filter-passing virus. The virus alone produced only a passing indisposition, whilst combined with the bacillus it caused a severe disease. Then Wilson Smith, Andrewes and Laidlaw (1933 and 1934) isolated a virus from a case of influenza which was capable of producing a febrile disease with involvement of the turbinates by intranasal inoculations in ferrets, the disease being transmissible in series from animal to animal. After numerous passages in the ferret it was found that the infection could be transmitted to mice. Similar findings have been obtained by Francis and Magill (1935) in America, by Burnet (1935) in Australia and by Hoyle and Fairbrother (1937).

Further experimental work has shown that many of the strains of viruses isolated from human cases of influenza have immunological relationships with the virus isolated from cases of swine influenza.

The outbreak of influenza during last winter gave us an opportunity of continuing the investigations commenced in 1933* on the isolation of filterable viruses from influenza cases, when it was found possible to grow in culture filter-passing microbes under anaërobic conditions. Here we propose to give some account of the study of the pathogenicity of two such viruses isolated from cases of influenza.

VIRUS P.M. 252/36.

This virus was obtained from a Berkefeld filtrate of lung extract from a case of acute influenza.† The filtrate was cultivated anaërobically in Hartley's broth with the addition of 10 p.c. horse-serum. There was no growth at 10 days, but at 26 days there was a diffuse cloudiness in the medium, whereas the uninoculated control was perfectly clear.

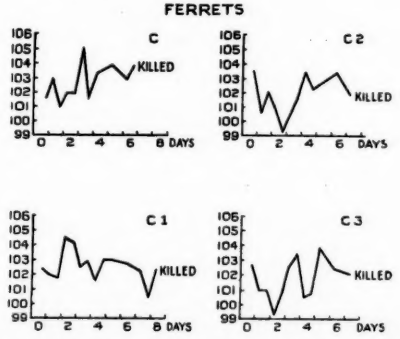

FIG. 1.—Temperature charts of ferrets, P.M. 252. Ferret C: Nasal instillation of anaërobic culture of Berkefeld filtrate of human lung extract. Ferret C1: Nasal instillation of turbinate extract from Ferret C. Ferret C2: Nasal instillation of lung extract from Ferret C. Ferret C3: Nasal instillation of Berkefeld filtrate of turbinate extract from Ferret C.

Transmission to Ferrets (Table I).

A ferret (Ferret C) was lightly anæsthetized with ether and about ¼ c.c. of the culture was dropped into the nostrils. Three days after inoculation the ferret developed a temperature of 105° F., which was followed by a fall to 101·6°, and then a continuous temperature of over 103° (Fig. 1). On the 6th day the ferret was killed and in the lungs were found dark hæmorrhagic areas in all lobes.

Three ferrets and 12 mice were inoculated with material from Ferret C. All inoculations were nasal instillations under light ether anæsthesia. Ferrets C1, C2 and C3 were inoculated with an extract of the turbinates, an extract

* McIntosh, 1933.

† Post-mortem No. 252/1936. A. S—, a male, aged 16 years. Died December 10th, 1936. Admitted to Middlesex Hospital December 10th, 1936, semi-comatose with a temperature of 104° F. Onset sudden 24 hours previously. *Pathological summary:* Influenzal pneumonia, hæmorrhagic œdema of both bases; severe tracheitis; enlarged spleen, 17 oz. Aërobic cultures showed the presence of numerous *Staphylococcus albus*.

of the lungs and a Berkefeld filtrate of the lungs respectively. Their temperature charts all showed, to a greater or less extent, the primary and secondary rise of Ferret C (Fig. 1). Ferrets C2 and C3 were killed on the 7th day, and Ferret C1 on the 8th day. In all three, dark areas of collapse and hæmorrhage were found in the lungs. The most extensive lesions were found in Ferret C3, where there were large deep purple areas throughout the lobes.

TABLE I.

Ferret.	Material.	Killed (days.)	Lung lesions.	Virulence. Turbinates.	Virulence. Lung.
C	26-day culture lung, P.M. 252	6	+	Ferret C1+, mice 7++, 7++, 7+, 7+,7+, 7−. (Berk.) ferret C3+	Ferret C2+, mice 7+, 7−, 7−, 7−, 7−, 7−
C1	Extract turbinates, Ferret C	8	+	..	..
C2	Lung extract, Ferret C	7	+	..	..
C3	Berkefeld filtrate turbinates, Ferret C	7	+	Ferret C4+, mice 9++, 9+, 9−, 9−	..
C4	Extract turbinates, Ferret C3	21	+	Mice 7++, 10+, 10+, 21+, 21+, 21−	Mice 7+, 21+, 21+, 21−, 21−. Berkefeld filtrate lungs, mice 8++, 21+, 21+, 21+, 21+, 21+

In the virulence tests in mice the amount of lung involvement found at autopsy is shown for each mouse by the ++, +, or − sign after the figure which indicates the day after inoculation on which the mouse died or was killed.

Transmission to Mice.

Six mice were inoculated with an extract of the lung of Ferret C. There were no deaths, and at the end of a week they were killed. In one mouse there was a large patch of hæmorrhagic consolidation in the right lower lobe and smaller patches elsewhere. The remaining 5 were negative.

Six mice were inoculated with an extract of the turbinates of Ferret C. One mouse died at the end of a week with complete hæmorrhagic consolidation of the lungs. The rest were killed, and in one a similar extensive lesion was found. In 3 others there were smaller lesions, and 1 was negative.

The details of the passage of this strain through mice are shown in Table II. Broth extracts or Berkefeld filtrates were used for passage. Berkefeld filtrates were used for the 2nd, 3rd, 6th, 9th and 10th passages. There was an increase in virulence for the first five passages followed by a fall at the sixth passage, where a Berkefeld filtrate was employed. The virulence then increased to the 8th passage, dropped slightly in the 9th, where a filtrate was used, and then suddenly became relatively avirulent. From the 13th to the 19th passage an attempt was made to resuscitate its virulence by rapid

TABLE II.—*Mouse Passage of Virus P.M. 252/36, Series A.*

Passage.	Interval (days).	Died (days).	Killed (days).
1	..	7++	7++, 7+, 7+, 7+, 7−
2*	7	3+, 7++	4+, 4+, 7+, 7+
3*	7	2+, 10++	8++, 8++, 17++
4	8	7++, 8++, 9++	9+, 9+, 9+
5	9	2+, 4+, 6+	6++, 6++, 6+
6*	6	1−	5+, 5+, 12++, 12+, 12+
7	12	2+, 7+	7++, 7+, 7+, 7−
8	7	5++, 5++, 5++, 5++	5++, 5++
9*	5	5++, 6++, 6++, 10+	12++, 12+
10*	6	..	12+, 12+, 12+, 12+, 12−
11	12	..	5+, 5+, 5−, 5−
12	5	..	2−, 2−, 7+, 7+, 7−, 7−
13	3	..	2+, 2−, 6+, 6+, 6+, 6−
14	2	..	2−, 2−, 6−, 6−, 6−
15	2	..	2+, 2−, 7+, 7+, 7−, 7−
16	2	..	2−, 2−, 13+, 13+, 13−
17	2	..	3−, 3−, 6+, 6−, 6−, 6−
18	3	..	2−, 2−, 14−, 14−, 14−
19	2	..	6+, 6+, 6+, 6+, 6+, 6+

* Berkefeld filtrate.

The figures and signs have the same significance as in Table I, except that the animals which died are shown separately.

TABLE III.—*Mouse Passage of Virus 252/36, Series B.*

Rabbit 372; 7th passage.
Killed 6 days — Lungs congested, dark red areas bases.
↓
Ferret D — Killed 8 days. Lungs voluminous, congested white spots, 1–3 mm. diam. on surface, turbinates swollen.
↓
Extract turbinates
↓
Ferret I — Killed 12 days. Lungs dark, congested, dark red patches left base.
↓
Extract turbinates and lungs
↓
Mice

Passage.	Interval (days).	Died (days).	Killed (days).
1	..	9−	11++, 11+, 11+, 11−, 11−
2	11	4++, 5++, 7++, 11+	11−, 11−
3	4	4++, 4++, 4+	9++, 9++, 9−
4	4	2?, 4++, 4++	4++, 4++, 4+
5	4	2++, 2++, 2++, 2++, 5++	5++
6	2	2++, 3++, 3++, 3++	4++, 4++
7	3	2++, 2++	7++, 7++, 7+, 7+
7*	3	3++, 4++, 5++, 7++, 7++	7+
8	2	5++, 5++	2++, 2++, 2++, 5−
9	2	3++, 5++, 6++, 6++	6++, 6−
10	6	4++, 4++, 4++, 4++	4−

* Berkefeld filtrate.

See Table I for the significance of the figures and signs.

passage, but without success. The loss of virulence can probably be accounted for by the long intervals between passages.

We were able, fortunately, to resuscitate this strain in mice from another source. The passage of this strain through rabbits will be referred to later. An extract of lungs from a rabbit in the 7th rabbit passage was inoculated to a ferret, which was killed at 8 days and showed lung lesions. An extract of this ferret's turbinates was inoculated to a second ferret, which, killed at 12 days,

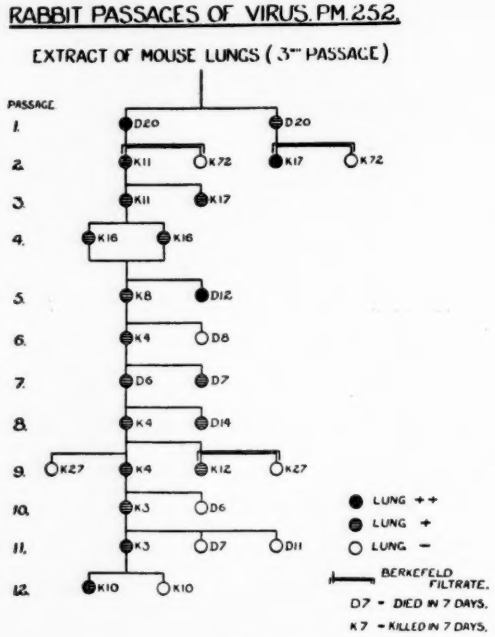

FIG. 2.

showed dark red patches in the lungs. Neither ferret had a typical temperature. A pooled extract of the turbinates and lung of the second ferret was inoculated to mice and the passage of this strain through mice is shown in Table III. This strain is very virulent, killing 50 p.c. of the inoculated mice in an average of 4½ days. Aërobic cultures of the lungs of these mice are almost invariably sterile. The ability of the virus to pass through a Berkefeld V filter seems to vary, probably owing to differences in the dilution and the amount of lung extract used. In the 7th passage a Berkefeld filtrate appeared to be even more active than the original lung extract. Lung extracts have also been virulent after being spun at 40,000 revolutions per minute for 7 minutes in the high-speed centrifuge—a speed sufficient to remove any bacteria from the supernatant fluid.

Transmission to Rabbits.

Two rabbits were inoculated intranasally with an extract of mouse lung from the 3rd mouse passage. There were no symptoms and no rise in temperature, but both rabbits were found dead 20 days after inoculation. There were dark red patches in the lungs of both. Berkefeld filtrates of their lungs were

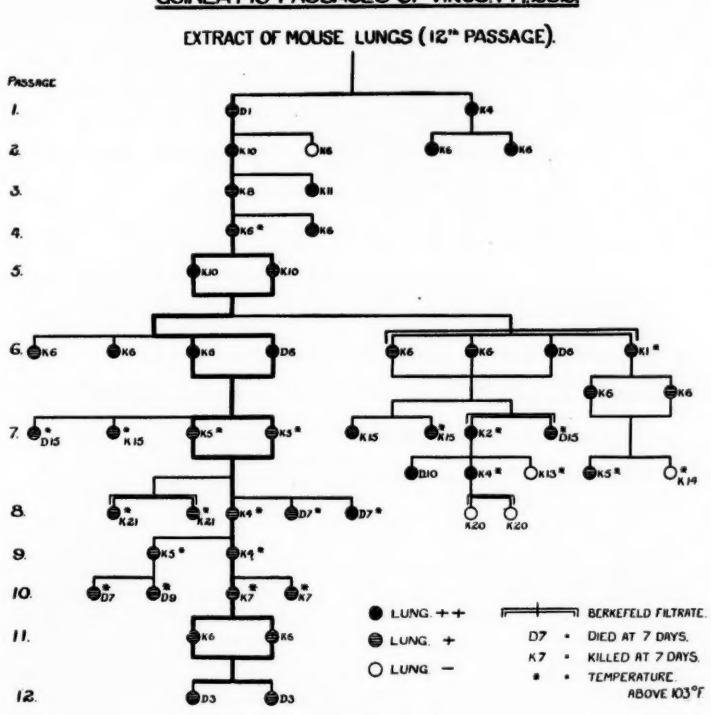

FIG. 3.

inoculated to 4 rabbits. One of these developed a temperature of 104·2° F. on the 10th day. It was killed on the following day and the typical deep red patches were found in the lungs. In all, 12 passages were made through rabbits (Fig. 2).

The symptoms and lesions were less evident in the rabbits than in other animals. A rise of temperature to over 103° F. was noted in 19 out of 29 rabbits. This was usually transient, and occurred at about the 9th or 10th day after inoculation. A rise to over 104° F. was observed in only 3 rabbits, in the 2nd, 11th and 12th passages respectively.

Transmission to Guinea-pigs.

Two guinea-pigs were inoculated intranasally with an extract of mouse lungs from the 12th mouse passage. One was found dead on the following day, and in the lungs there were irregular hæmorrhagic patches. Twelve successful passages of this virus were made through guinea-pigs and the details

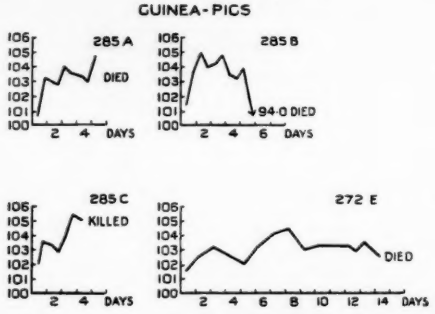

FIG. 4.—Temperature charts of guinea-pigs, virus P.M. 252/36. Guinea-pigs 285A, 285B, 285C: 8th guinea-pig passage. Guinea-pig 272E: 7th guinea-pig passage.

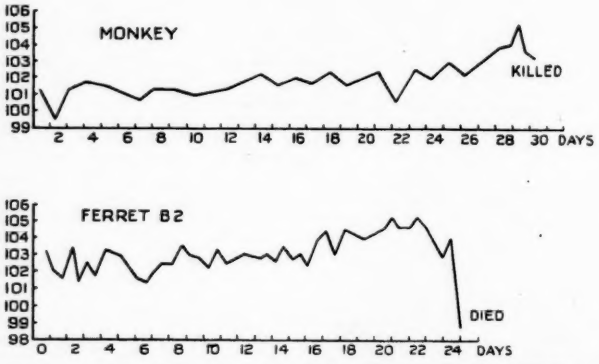

FIG. 5.—Temperature chart of monkey, virus P.M. 252/36. Nasal instillation of extract mouse lung from mice of 6th mouse passage. Temperature chart of Ferret B.2. P.M. 1/37. Nasal instillation of lung extract from Ferret B.

of these passages are given in Fig. 3. It was successfully transmitted by a Berkefeld filtrate through two passages (6th and 7th). A further attempt in the 9th passage apparently failed in that there was no rise in temperature and no naked-eye lung lesions in the two guinea-pigs inoculated. Microscopically, however, there were quite definite influenzal lesions in the lungs of both.

Except for one guinea-pig in the 4th passage and one in the 6th passage, temperatures of over 103° F. were not observed till the 7th passage. Five of

the guinea-pigs in the 7th passage, all inoculated with lung extract, developed temperatures of over 104° F. from the 4th to the 8th day after inoculation. In the next three passages a high temperature was frequently recorded. In 14 guinea-pigs inoculated with extract, temperatures of over 104° F. were noted in 9 and temperatures of over 105° F. were noted in 6. The temperature charts of 4 guinea-pigs are shown in Fig. 4. Deaths also became more frequent in the later passages, indicating an increase of virulence for guinea-pigs.

Transmission to a Monkey.

A cercopithecus monkey was inoculated intranasally with an extract of mouse lung from the 6th mouse passage. The temperature chart of this monkey is shown in Fig. 5. During the first fortnight the temperature remained normal, but during the third week it was about a degree higher. There was then a gradual rise to 105·2° F. on the 29th day. The monkey was killed 30 days after inoculation.

The lungs were voluminous and of a dark grey colour with emphysematous margins. The upper part of the left lower lobe was occupied by a large firm nodule of a deep purplish red. There was a similar but much smaller nodule on the posterior surface of the lower right lobe.

Serological Tests.

Protection.—Three neutralization tests with human convalescent serum and with the Hampstead $1H_2$ anti-influenzal horse-serum* showed some evidence

TABLE IV.—*Protection Experiments. Virus P.M. 252/36.*

Extract Lungs 5th Mouse Passage. 37° C.; ½ hour.

	Died (days).		Killed (days).
āā saline 6 mice . .	5+, 5++, 7++, 10++, 10++, 11++	.	..
āā normal human serum—6 mice	1 ?, 1+, 1+, 6+, 11+	.	13−
āā convalescent serum—6 mice	9++, 9++, 11+, 11+	.	13+, 13−

Berkefeld Filtrate Lungs 8th Mouse Passage. 37° C.; ½ hour.

	Died (days).		Killed (days).
āā saline—6 mice . .	5++, 6++, 6++, 10+	.	12++, 12+
āā $1H_2$ serum—6 mice .	9 ?	.	12+, 12+, 12−, 12−, 12−

Extract Lungs 10th Mouse Passage of Resuscitated Virus (Table III) Spun at 40,000 r.p.m. for 7 min. Room Temperature; 15 min.

	Died (days).		Killed (days).
āā saline—6 mice . .	3++, 3++, 4+, 4+		7++, 7++
āā $1H_2$ serum 1/10—6 mice	5+, 6+	.	7++, 7+, 7−, 7−
āā convalescent serum 1/5 6 mice	3+	.	7++, 7−, 7−, 7−, 7−
āā normal serum 1/5—6 mice	..	.	7++, 7++, 7++, 7++, 7+, 7+

* For this sample of serum we are indebted to Dr. C. H. Andrewes.

of protection in mice—see Table IV. Deaths were fewer and later, and the lung lesions were less extensive in the mice inoculated with the immune serum mixtures. This effect was most evident with convalescent serum.

Complement-fixation.—An antigen consisting of dried rabbit lung from the first passage in rabbits gave a definite fixation of complement with all of several samples of human convalescent serum, and practically none with a pooled sample of normal human sera.

VIRUS P.M. 1/37.

A Berkefeld filtrate of the human lung extract* was inoculated intranasally to 2 ferrets and to 6 mice. One ferret (Ferret B) developed a temperature of 103·8° F. on the 4th day and a secondary rise on the 6th day after inoculation. It was killed on the 7th day, and had deep red hæmorrhagic areas in both lungs. The second ferret (Ferret A) did not have a rise in temperature during the first week. Apart from a temperature of 104·8° F. on the 9th day there was nothing to note for 23 days. From then to the 29th day there was a step-like rise to 105° F. During the next 2 days the temperature fell to 103°, and on the 32nd day the ferret was killed. There were numerous dark red consolidated areas in both lungs. The second passage in ferrets and the virulence of the turbinates and lung extracts for mice are

TABLE V.—*Ferrets P.M. 1.*

Ferret.	Material.	Died or killed (days)	Lung lesions.	Virulence. Turbinates.	Virulence. Lung.
A	Berkefeld filtrate lung P.M. 1	K32	+	Mice 16+, 16+, 16−, 23+, 23+, 23−	Mice 13+, 16+, 16+, 23+, 23−, 23−
B	Ditto	K7	+	Ferret B1+; mice 6 ?, 6+, 6+, 6−, 6−, 6−	Ferret B2+; mice 6+, 6+, 6−, 6−, 6−
B1	Extract turbinates Ferret B	K20	+	Mice 7++, 14++, 14+, 14+, 14+ 14−	Mice 14+, 14+, 14+, 14+, 14+, 14+
B2	Extract lung, Ferret B	D25	+	..	..

shown in Table V. The mice were inoculated with human lung filtrate and were killed at the end of a week. In the lungs of one there was plum-coloured consolidation of all the lobes and in another there were small hæmorrhagic patches. The remainder showed no lung lesions. The details of the passage of this strain through mice is shown in Table VI. The virulence increased up to the third passage, where a Berkefeld filtrate was at least as virulent as the original extract. In the sixth passage there were no deaths, but lung lesions were found in 5 of the mice when they were killed at 12 days. The lungs of these mice were dried and stored in the ice-chest. Two months later an attempt

* P.M. No. 1/1937. R. F—, male, aged 19 years, died January 1st, 1937, after a history of 5 days' illness. Diabetic treated with protamine insulin. *Pathological summary:* Influenzal pneumonia, tracheitis, fatty liver. Cultures from the lungs grew numerous *Staphylococcus albus.*

was made to resuscitate the virulence of this strain from the dried material by rapid passage. Passages were made three times a week for 8 passages, but the virulence of the strain did not increase sufficiently to cause the death of the mice, although lesions were frequently found when the mice were killed. The temperature chart of Ferret B2 is shown in Fig. 5.

White cell counts on the mice of the third passage showed a relative increase in mononuclear cells and a relative decrease in polymorphonuclear leucocytes. The average total white cell counts of 3 of the mice inoculated with extract and of 3 of the mice inoculated with filtrate did not vary between the first and

TABLE VI.—*Mouse Passages of P.M. 1/37.*

Passage.	Interval (days).	Died (days).	Killed (days).
1*	..	..	7++, 7+, 7−, 7−, 7−, 7−
2	7	2+, 6+	6++, 6++, 6+, 6+
3	6	7+, 7+, 12+	4+, 7+, 24+
3*	..	8+, 10+, 13++, 13+	4+, 4+
4	4	17+, 20++	6+, 6+, 6+, 6+
5	6	1+, 6++, 8+	8+, 8+, 8+
6	8	..	12++, 12++, 12++, 12+, 12−

* Berkefeld filtrate.

third day after inoculation, being 22,000 per c.mm. on both occasions. On the other hand, the average percentages of lymphocytes and mononuclears in all 12 mice increased from 38 and 9 to 45 and 21·5 respectively, whereas the polymorphonuclear leucocytes decreased from 53 to 33·5 p.c.

PATHOLOGICAL LESIONS.

When those animals which died as a result of the inoculation were examined, striking changes were found in the lungs. In these organs there were large areas of collapse and hæmorrhage. Microscopically proliferative changes in the lungs were shown by a great increase in the numbers of mononuclear cells which caused thickening of the alveolar walls and consolidation, producing what might be termed an interstitial pneumonia. At the same time there was an increase in the mononuclear cells of the blood. In a certain number the liver was enlarged and fatty. Other changes have been found from time to time, but these will be described at a later date.

DISCUSSION.

From the experiments detailed above it appears that viruses can be obtained from filtered lung extracts of cases of human influenza capable of causing infection in laboratory animals. Further it would appear that these viruses are partially neutralized by convalescent influenza sera, and some degree of protection was afforded by an immune serum ($1H_2$) prepared against the influenza virus of W. Smith, Andrewes and Laidlaw. But much more work

has to be done on the relationship of these various viruses before their correct relationship is known. The evolution of a typical disease seems much slower with our strains than with those of W. Smith, Andrewes and Laidlaw. In the ferret there is the usual initial rise of temperature in the first few days after the inoculation, but the secondary rise is delayed till the 10th to 14th day or even longer. It is at this period that the characteristic lung changes are most definite. But as these writers have made a point of carrying on the passages at short intervals, their virus may have adapted itself to such a procedure. In their case a local and superficial proliferation of the virus occurs, whilst we, on the other hand, have allowed time for a more general invasion of the tissues, and particularly those of the lungs.

SUMMARY.

1. Two strains of virus isolated from the lungs of cases of human influenza were found capable of causing an experimental disease with lung lesions in animals.

2. The lesions were produced in ferrets, mice, rabbits, guinea-pigs and a monkey by intranasal inoculation under anæsthesia.

3. Preliminary immunological tests show definite protection by convalescent influenza sera and by the anti-influenza serum $1H_2$ prepared by Smith, Andrewes and Laidlaw against their influenza virus.

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